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Streptococcus pneumoniae and the host: activation, evasion and modulation of the human innate immune system

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Stockholm 2016

Cover: Fluorescent microscopy image of dendritic cells and *Streptococcus pneumoniae* serotype 4 mutant T4RΔ*ply*. Dendritic cells are stained for actin with Rhodamine Phalloidin (red), nuclei are stained with DAPI (blue) and bacteria are labeled with FITC (green).

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Published by Karolinska Institutet.
Printed by AJ Eprint AG, 2016

ISBN 978-91-7676-464-0

Institutionen för Mikrobiologi Tumör- och Cellbiologi

Streptococcus pneumoniae and the host: activation, evasion and modulation of the human innate immune system

AKADEMISK AVHANDLING

som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras i **Inghesalen, Tomtebodavägen 18A**, Karolinska Institutet Solna.

Fredagen den 2 december 2016, kl. 09.00

av

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ABSTRACT

Streptococcus pneumoniae is a major cause of severe infections such as pneumonia, septicemia and meningitis, but also a common colonizer of the nasopharynx in children. In most individuals colonization is harmless and eventually cleared by the immune system, but in rare cases pneumococci can reach deeper into the body and cause diseases. It is not understood why pneumococci cause infections in a few individuals while in most cases the bacteria are limited to the nasopharynx and eventually cleared. It is clear, however, that a well-orchestrated immune system is essential to prevent and limit pneumococcal infections. Macrophages are essential for an early clearance of pneumococci and dendritic cells are required to initiate appropriate adaptive responses. Both cell types were studied in this thesis.

Cytokine secretion by dendritic cells directs the development of T-cells, and we studied the induction of IL-12 secretion by dendritic cells in response to pneumococci. We showed that pneumococcal RNA was recognized by TLR3, which together with the adapter molecule TRIF induced secretion of IL-12. Infection of dendritic cells with influenza A virus upregulated TLR3 expression which contributed to a more efficient detection of pneumococci and enhanced IL-12 secretion.

We observed that the pneumococcal pore forming toxin pneumolysin had profound effects on cytokine responses in human dendritic cells and macrophages. We found a cell death independent inhibition of cytokine secretion in human dendritic cells and macrophages by pneumolysin expressing pneumococci. Interestingly however, cytokine secretion by macrophages derived from the human THP-1 cell line was enhanced in the presence of pneumolysin. We described pneumolysin mediated effects on these cell types and explored initial insight into the underlying mechanisms.

Clearance of pneumococci by macrophages is supported by deposition of complement on the bacterial surface. The pneumococcal surface protein PspC binds human Factor H to evade opsonophagocytosis, and can also act as an adhesin. We characterized two variants of PspC proteins present in B6 clinical isolates. The two proteins showed differential expression patters on the bacterial surface and had distinct functions as Factor H binding protein or adhesin. Small changes in surface localization impaired the protein function, indicating the importance of correct surface expression.

We tested the effects of vitamin D on the activation of dendritic cells by pneumococci and the induction of T-cell responses. Vitamin D supported dendritic cell maturation and skewed T-cell responses from an inflammatory to a regulatory phenotype.

This work gives insight into the complex interactions between *S. pneumoniae* and human immune cells, and underlines the dynamic effects of pneumococcal virulence factors on the host. A thorough understanding of the activation and evasion of immune responses by pneumococci as well as the effects of immunomodulatory agents such as vitamin D is essential for the development of future treatment options and vaccine approaches.

LIST OF SCIENTIFIC PAPERS

This thesis is based upon the following papers, which will be referred to by their Roman numerals throughout this thesis:

I. **Laura Spelmink**, Vicky Sender, Karina Hentrich, Thomas Kuri, Laura Plant*, Birgitta Henriques-Normark*
Toll-like receptor 3/TRIF-dependent IL-12p70 secretion mediated by *Streptococcus pneumoniae* RNA and its priming by influenza A virus coinfection in human dendritic cells
Mbio, 2016, vol. 7, p. e00168-16

II. **Laura Spelmink**, Karthik Subramanian, Susan Farmand, Giorgia Dalla Libera Marchiorini, Laura Plant, Birgitta Henriques-Normark
Pneumococcal toxin pneumolysin mediates cell type specific inhibition of cytokine secretion
Manuscript

III. Anuj Pathak, Vicky Sender, **Laura Spelmink**, Jan Bergstrand, Jerker Widengren, Birgitta Henriques-Normark
Spatial representation and density of human factor H binding proteins on *Streptococcus pneumoniae* affects virulence function
Manuscript

IV. Marie Olliver, **Laura Spelmink**, Jeffni Hiew, Ulf Meyer-Hoffert, Birgitta Henriques-Normark*, Peter Bergman*
Immunomodulatory effects of vitamin D on innate and adaptive immune responses to *Streptococcus pneumoniae*
The Jounal of Infectious Diseases, 2013, v. 208 (9), p. 1474-1481

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CONTENTS

LIST OF ABBREVIATIONS

AIM2	absent in melanoma 2
AP-1	activating factor-1
APCs	antigen presenting cells
ASC	associated speck-like protein containing a caspase activation and recruitment domain
CbpA	choline binding protein A
CC	clonal complex
CD	cluster of differentiation
DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
GAS	IFN-γ activated site
GlcNAc	N-acetylglucosamine
GM-CSF	granulocyte-macrophage colony-stimulating factor
hBD-3	human beta defensin 3
HEK294	Human embryonic kidney 293
Hic	Factor H inhibitor of complement
IAV	influenza A virus
IFN	interferon
Ig	immunoglobulin
IKK	IκB kinase
IL	interleukin
IRAK	interleukin-1 receptor-associated kinase
IRF	interferon regulatory factor
ISGF3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated response element
IκB	inhibitor of NFκB
JAK	Janus kinase
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MAC	membrane attack complex
MAPK	mitogen-activated protein kinase
MARCO	macrophage receptor with collagenous structure
MAVS	mitochondrial antiviral signaling protein
MBL	mannose binding lectin
M-CSF	macrophage colony-stimulating factor
MDA-5	melanoma differentiation-associated protein 5
MDCK	Madine-Darby canine kidney
MDP	muramyl dipeptide
MHCII	major histocompatibility complex class II
MRC-1	macrophage mannose receptor 1
mRNA	messenger RNA
MurNAc	N-acetylmuramic acid
MyD88	myeloid differentiation primary response protein 8
NET	neutrophil extracellular trap
NFκB	nuclear factor κB
NLR	NOD-like receptor
NOD	nucleotide-binding oligomerization domain

PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cells
Pbp	penicillin binding protein
PCV	pneumococcal conjugate vaccine
pIgR	poly Ig receptor
PMA	phorbol myristate acetate
Poly I:C	Polyinosinic-polycytidylic acid
PPV	pneumococcal polysaccharide vaccine
PRR	pattern recognition receptor
PspC	pneumococcal surface protein C
RCT	randomized placebo controlled trail
RIG-I	retinoic acid-inducible gene 1
RIP	receptor interacting protein
RLR	RIG-I-like receptor
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal RNA
RTI	respiratory tract infections
RXR	retinoid X receptor
SC	secretory component
SIGNR1	SIGN related-1
siRNA	small interfering RNA
SOCS1	suppressor of cytokine signaling 1
SpsA	Streptococcus pneumoniae secretory IgA binding protein
SR-A	class A macrophage scavenger receptor
ssRNA	single stranded RNA
STAT	Signal Transducers and Activators of Transcription
STING	Stimulator of IFN genes
TA	Teichoic acid
TAK1	transforming growth factor-b-activated protein kinase 1
TBK1	TANK-binding kinase
TGFβ	transforming growth factor beta
T_H	helper T-cell
TIR	Toll/interleukin-1 receptor
TIRAP	TIR-domain containing adapter protein
TLR	Toll-like receptors
TNFα	tumor necrosis factor
TRAF6	tumor necrosis factor receptor-associated factor 6
TRAM	TRIF-related adapter molecule
Treg	regulatory T-cell
TRIF	TIR-domain-containing adapter inducing IFNβ
tRNA	transfer RNA
TYK2	tyrosine kinase 2
UV	ultraviolet
VDR	vitamin D receptor
VDRE	vitamin D response element
WTA	wall teichoic acids

1 INTRODUCTION

1.1 *STREPTOCOCCUS PNEUMONIAE*

Streptococcus pneumoniae was first described in 1881 when Steinberg and Pasteur independently reported the isolation of a lancet shaped diplococcus from the blood of rabbits injected with human saliva (1, 2). Within the same decade, the potential of the bacterium to cause pneumonia, meningitis and otitis media was established and due to its role in pneumonia, the bacterium was referred to as *Pneumococcus* or *Diplococcus pneumonia*e. In 1974 it was given its current name, *Streptococcus pneumoniae*, based on the characteristic long chains of cocci that are formed when the bacterium grows in liquid media (3). Nevertheless, the bacterium is still commonly referred to as the pneumococcus.

The pneumococcus is facultative anaerobe and grows on blood agar plates where it forms colonies surrounded by a green zone, indicating α-hemolysis (Fig. 1). The green color appears because the bacterium lyses red blood cells and oxidizes hemoglobin. *S. pneumoniae* is sensitive to optochin and can thereby be distinguished from bacteria of the commensal *S. viridans* group, which also are α-hemolytic.

The Gram-positive cell wall of pneumococci is surrounded by a characteristic thick polysaccharide capsule. The composition of the capsular polysaccharides is very diverse and determines the serotype of a pneumococcus. Over 90 different serotypes have been identified so far.

Figure 1 Serotype 4 strain TIGR4 grown over night at 37°C and 5% CO_2 on a blood agar plate.

Pneumococci are naturally transformable, which means that they efficiently take up genetic material from their environment and integrate it into their genome, creating a high genetic diversity between pneumococcal strains (4). Griffith demonstrated this for the first time by injecting mice subcutaneously with an unencapsulated non-virulent variant, as well as a heat killed encapsulated variant of *S. pneumoniae.* The mice succumbed to the infection and Griffith could isolate encapsulated bacteria from the blood, indicating that the genetic material for the capsule was transferred from the dead bacteria to the live and previously unencapsulated ones (5). This led later to the groundbreaking discovery of *deoxyribonucleic acid* (DNA) as the transforming principle by Avery, which was the first time DNA was identified as genetic material (6).

1.1.1 Pneumococcal Diseases

Colonization

S. pneumoniae is part of the natural flora of the human nasopharynx and small children are commonly colonized with the bacterium. Pneumococci are airborne, spread via droplets, and colonization rates can reach up to 60% in children (7, 8), whereas around 5% of adults are colonized (9, 10). In most cases, the bacterium resides silently in the nose and is eventually cleared by the immune system, but in rare cases pneumococci reach deeper into the body and cause pneumococcal diseases.

The serotypes of pneumococci differ in their potential to colonize the nose and to cause invasive disease. While some serotypes, such as 6B, 19F and 23F are frequent colonizers and rarely cause disease, others, such as serotype 1, 5 and 7 are prominent causes of disease (11).

Carriage duration varies between serotypes and age groups. A Swedish study observed periods of carriage between 2 and 368 days, with an average duration of 37 days. The duration of colonization also depended on the age, where children under the age of 5 had significantly longer periods of colonization than older individuals. Serotype 6 and 23 showed the longest colonization periods in children younger than 5 years (12).

Pneumococcal colonization is a prerequisite for pneumococcal disease and can lead to mild diseases such as otitis media and sinusitis or severe invasive diseases like pneumonia, bacteremia and meningitis.

Otitis Media

The most common manifestation of *S. pneumoniae* infections is acute otitis media, an infection of the middle ear which occurs with high frequency in small children. In the United States, pneumococcal infections are estimated to annually cause 3.1 million cases of otitis media in children younger than 5 years (13). The infection usually fully resolves spontaneously but recurrent otitis media can lead to sequelae including hearing loss and speech delay. Pneumococci rank among the most frequent bacteria isolated in otitis media (14) and are associated with early acute otitis media. These early infections can predispose children to infections with other bacteria and viruses leading to recurrent and more persistent mixed-species infections (15).

Sinusitis

Sinusitis, also known as rhinosinusitis, is an inflammation of the paranasal sinuses, which are cavities in the cranial bone around the nose. *S. pneumoniae* is one of the most frequently isolated bacteria causing sinusitis (16).

Pneumonia

Pneumonia, an inflammatory condition of the lungs, is the second most common pneumococcal disease. Community acquired pneumonia is common in children under 5 years and in adults older than 65 years (17). It is the cause of 19% of the deaths worldwide in children under 5 years, which makes it the biggest killer of this age group. Death due to pneumonia varies strongly between regions, with 2% of childhood deaths caused by pneumonia in the industrial world and 20% in developing countries (18). In almost all countries of the world *S. pneumoniae* in the leading cause of pneumonia (18) and in Europe 35% of the pneumonia cases are caused by this bacterium (17).

A few serotypes were shown to have a high potential to cause pneumonia, such as serotype 1 and 5. There is also a correlation between the risk for death from pneumonia and the carriage prevalence of serotypes, as well as an inverted relationship between the carriage prevalence and invasive pneumonia. Serotype 19F, for example, has a high carriage rate and is associated with a high risk of death due to pneumonia, but the potential of 19F to cause pneumonia is very low. Serotype 1 in contrast, has a low carriage rate and causes a low risk of death by pneumonia, but its potential to cause pneumonia is very high (19). Short-term mortality (within 30 days) of hospitalized pneumococcal pneumonia patients ranges from 4-18% (17).

Bacteremia and Sepsis

Bacteremia occurs when pneumococci infect the blood stream. This can happen in connection with otitis media, pneumonia or meningitis, or without a focal infection. The bacteria can cause a strong immune response in the body leading to the development of sepsis. The 30-day mortality of sepsis is around 20% depending on the severity of the sepsis, and the age of the patients (20-22). The serotype also contributes to the severity of the infection and there is an inverse relationship between the invasive disease potential and the disease severity as well as fatality rate of the serotypes (23).

Meningitis

Meningitis is an inflammation of the meninges, which are the membranes covering the brain and the spinal cord. Meningitis is a severe disease with 16-37 % mortality and common long-lasting neurological sequelae, affecting 30-52 % of the survivors. Sequelae include hearing loss, cognitive impairment and neurological deficits (24). *S. pneumoniae* is the main cause of meningitis in most of the world and it especially affects children younger than 2 years of age (25). In the United States, 2000 cases of pneumococcal meningitis are reported annually (13).

The global burden of pneumococcal disease

Infections with *S. pneumoniae* contribute strongly to the global mortality. It was estimated for the year 2000 that pneumococcal diseases caused 800,000 deaths in children under the age of 5 years which was 11% of all deaths in this age group (26). In 2008 it was estimated that pneumococcal infections were responsible for 500,000 deaths in children younger than 5 years, which was 5% of the total deaths in this age group (27). The mortality due to pneumococcal disease varies largely between countries with low mortality in the developed world and higher mortality in the less developed countries. The highest mortality in children under 5 years can be found in south Asia and sub-Saharan Africa (Fig. 2). In the EU, the rate of reported invasive pneumococcal diseases decreased 2010 to 2014 from 6.0 to 4.8 per 100,000 people and the rates for the age groups under 1 year and over 65 years in 2014 were 11.3 and 13.8 per 100,000, respectively (28).

Clearly, the number of pneumococcal infections and the associated mortality is decreasing worldwide. The developed world has access to vaccines and optimal treatment in hospitals which keeps the case and mortality rates of pneumococcal infections very low. Especially in south Asia and sub-Saharan Africa where case and mortality rates are high, prevention and treatment of pneumococcal infections requires significant improvement.

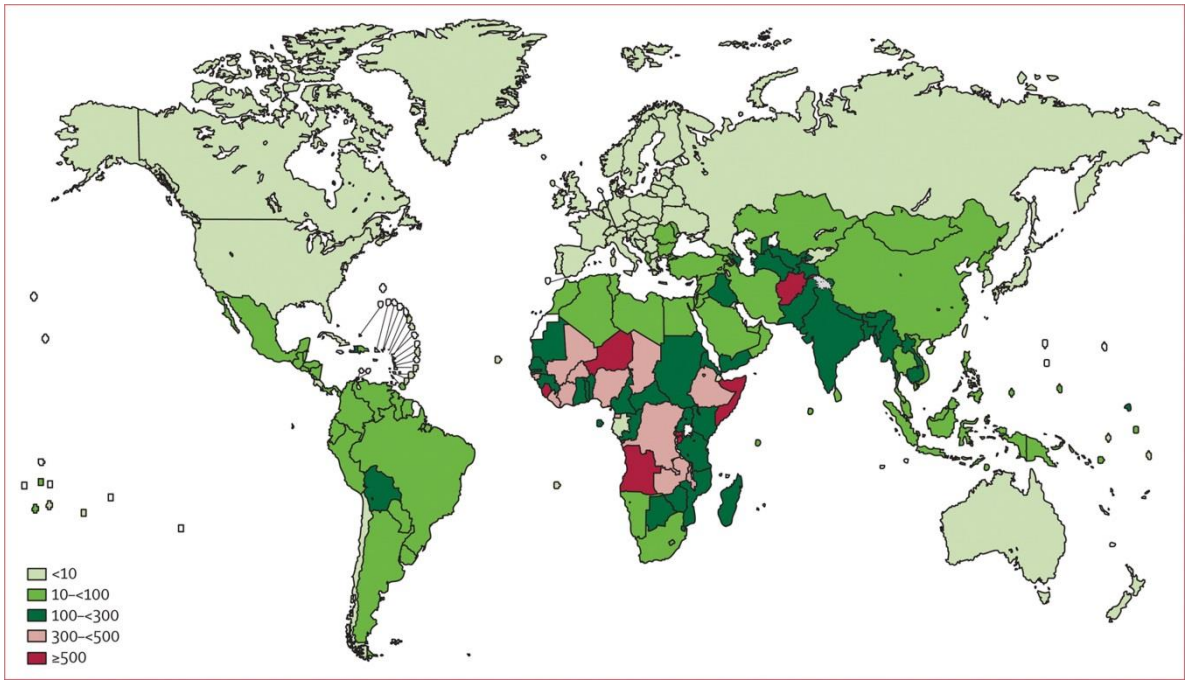

Figure 2 Global mortality rates of pneumococcal disease in children younger than 5 years. Estimated mortality rates are shown per 100,000 children younger than 5 years. Adopted from (26).

1.1.2 Risk Factors

Several risk factors for pneumococcal diseases have been identified. A functional immune system is key to prevent and clear pneumococcal infections. While adults with a functional immune system rarely suffer from pneumococcal infections, the immune system of children under the age of 2 years is not fully matured and the immune responses in the elderly weaken, which puts these age groups at an increased risk to acquire pneumococcal infections. Understandably, immunocompromised individuals (due to e.g. HIV, cancer, primary immune deficiencies, immunosuppressive therapy or splenectomy) are also at high risk for pneumococcal disease (29, 30).

Risk factors for immunocompetent individuals are underlying diseases, including diabetes, cardiovascular diseases and alcoholism (29, 30). Additionally, ethnic groups such as Afro-Americans, Native Americans and Alaskan native populations have higher risks for colonization, which indicates a genetic factor (31). Behavioral factors such as smoking, as well as socioeconomic and environmental factors, including crowding, contact with children, or preceding viral infections also increase the risk for pneumococcal infections (29, 30).

Coinfections with Influenza A virus

Infections with influenza predispose individuals for severe secondary pneumococcal infections. A recent study showed that bacterial superinfection in hospitalized influenza patients occurs in 2% to 65% of the cases, and *S. pneumoniae* was the most isolated bacterium (32). The impact of superinfections with *S. pneumoniae* becomes particularly clear during pandemic influenza outbreaks, like the Spanish flu in 1918, the Asian flu in 1957, the Hong Kong flu in 1968 and the recent "swine flu" in 2009 (33). The Spanish flu in 1918 was caused by an influenza A H1N1 virus and caused over 50 million deaths worldwide. Only a small proportion (5%) of the deaths occurred early after infection, while most occurred 7-14 days after infection. This, together with the isolation of bacteria, mainly *S. pneumoniae*, in 85-90% of the autopsies indicates that bacterial superinfection was a leading cause of death during this pandemic (33, 34). The pandemics in 1957 and 1968, caused by the H2N2 and H3N2 viruses, respectively, had much lower mortality due to the use of antibiotics and influenza vaccines. Nevertheless, *Staphylococcus aureus* was the main bacterium isolated during the 1957 flu and *S. pneumoniae* during the 1968 flu. The "swine influenza" caused by an H1N1 virus in 2009 resulted in 200,000 estimated deaths, which is not higher than during seasonal influenzas. However, the affected age group was younger than during a seasonal influenza. Bacteria were isolated from 25-50% of the severe infections and *S. aureus* and *S. pneumoniae* were most commonly found (33, 35, 36).

1.1.3 Prevention and Treatment

Treatment

Pneumococci are naturally sensitive to penicillin, therefore penicillin and other β-lactams are the antibiotics of choice to treat pneumococcal infections. These antibiotics bind to *penicillin binding proteins* (Pbp) which are important for cell wall synthesis, leading to death and lysis of the bacteria.

Penicillin was first introduced in 1943 and since then has also been used to treat pneumococcal infections. Penicillin use has dramatically improved disease outcome for patients and decreased the mortality for pneumococcal sepsis from 82% to 17 % (37). However, antibiotic resistance within pneumococcal isolates emerged soon, and the first penicillin resistant strain was isolated in Australia in 1967 (38). Since then penicillin and β-lactam resistance has dramatically increased, and up to 50% of the pneumococcal isolates have reduced susceptibility to penicillin in some regions. In countries with low antibiotic use, like Sweden, resistance rates are low. In 2014 7.9 % of invasive pneumococcal isolates in Sweden had reduced susceptibility to penicillin (39).

Resistance is mediated by allelic variants of Pbps with low affinity for β-lactam. The *pbp* genes of highly resistant strains have a mosaic structure and have probably evolved as a consequence of point mutations as well as recombination with genes from the oral commensal bacteria *Streptococcus mitis* and *Streptococcus oralis* which were acquired by horizontal gene transfer (4).

Infections with β-lactam resistant pneumococci are treated with macrolides or fluoroquinolones. Macrolides inhibit protein synthesis by binding to a ribosomal subunit, which prevents binding of the ribosome to the *messenger ribonucleic acid* (mRNA). Fluoroquinolones act on the enzyme topoisomerase which is involved in DNA synthesis. Strains resistant to macrolides or resistant to both penicillin and macrolides are frequently isolated in European countries (39).

Prevention

The pneumococcal vaccines currently on the market are listed in Table 1. The *23-valent pneumococcal polysaccharide vaccine* (PPV23) contains polysaccharides of the pneumococcal capsule and protects against the 23 most common serotypes causing invasive disease. The vaccine was introduced in 1983 but due to the low immunogenicity of pure polysaccharides, it did not induce sufficient immunity in children under 2 years (29, 30). Nevertheless, PPV23 is recommended for individuals over 65 years.

Table 1 Pneumococcal vaccines currently on the market

Vaccine	Manufacturer	Serotypes covered	Available since
PPV23 - Pneumovax®	Merck	1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, 33F	1983
PCV7 - Prevenar®	Pfizer	4, 6B, 9V, 14, 18C, 19F, 23F	2000
PCV10 - Synflorix®	GSK Biologicals	**1**, 4, **5**, 6B, **7F**, 9V, 14, 18C, 19F, 23F	2009
PCV13 - Prevenar13®	Pfizer	1, **3**, 4, 5, **6A**, 6B, 7F, 9V, 14, 18C, **19A**, 19F, 23F	2010

In 2000, the first *pneumococcal conjugate vaccine* (PCV) was licensed. This vaccine contains polysaccharides conjugated to a non-toxic recombinant variant of diphtheria toxin, which improves immunogenicity. PCVs are able to induce T-cell dependent B-cell responses and long lasting immunity in children younger than 2 years (described further in chapter 1.2.2). In PCV7, 7 capsular serotypes are included and they were chosen based on the most common serotypes causing invasive disease in the United States. The serotype distribution varies among countries and the PCV7 vaccine covered the serotypes of 70-88 % of all invasive pneumococcal diseases in children in North America, Europe and Africa, but fewer than 65% in Latin America and Asia (40).

In 2009 and 2010 the new conjugate vaccines PCV10 and PVC13 were introduced. The additional serotypes in these vaccines should account for global differences in in coverage. The PCV10 and PCV13 vaccine should prevent acute otitis media, pneumonia and invasive pneumococcal disease in children under 5 and PCV 13 can also be used in older age groups (29, 30).

In 2012 44% of all WHO member states had introduced PCVs in their childhood vaccination program (29, 30). The PCVs have globally dramatically reduced invasive pneumococcal diseases among all age groups (41). In the United States, the invasive pneumococcal disease cases in children under 5 years decreased 77% after the introduction of PCV7 and the rate of hospitalization for pneumococcal pneumonia in children under 2 years decreased 65% (42, 43). Additionally, carriage rate of pneumococci and the frequency of antibiotic resistant strains decreased in some countries (13), whereas other countries found the same rates of carriage and antibiotic resistance after vaccine introduction (44). In some countries the introduction of PCV7 also reduced pneumococcal disease in the un-vaccinated population, such as adults under 65 years (44) and children under 90 days of age (45). This "herd effect" of vaccines is especially important to protect groups which cannot be vaccinated, such as the smallest children.

Although PCV7 had positive effects on pneumococcal disease globally, it also led to the emergence of serotypes not covered by the vaccine, so called non-vaccine types, especially serotype 19A (46, 47). The inclusion of 19A in PCV13 counteracted this emergence but did not prevent from the emergence of further serotypes not covered by the 13-valent vaccine. In the Stockholm area an increase in carriage of the non-vaccine types 11A and 22F has been observed during the last years after the introduction of PCVs (44).

It is not fully understood which processes underlie the emergence of non-vaccine types, but most likely the elimination of vaccine strains gives non-vaccine types the possibility to take over the free niche. Another explanation is that strains that were successful prior to vaccination switch their capsular type by acquiring capsule genes over horizontal gene transfer from co-colonizing strains.

Future vaccines should offer protection from a larger spectrum of pneumococci. The number of serotypes that can be included in a PCV is limited and other vaccine approaches are being investigated. Current research is focused on vaccine candidates for a protein vaccine. The optimal protein should be a surface exposed virulence factor present in all virulent strains. Several proteins have been implicated and are currently studied, among them are pneumolysin and *pneumococcal surface protein C* (PspC) (48, 49), which are studied in this thesis. Since it is easier for a bacterium to evade a vaccine composed of one or a few proteins, another promising approach is the use of a whole cell vaccine composed of killed non-encapsulated pneumococci (50).

1.2 THE IMMUNE SYSTEM

Our body is under constant attack by potentially infectious agents such as bacteria, viruses, fungi and parasites, and the immune system prevents and eliminates these infections. The immune system is highly complex and includes physical barriers, lymphoid organs, immune cells as well as soluble mediators. The cells of the immune system communicate by direct cell contact, or secretion of molecules such as cytokines and chemokines that can modulate and regulate the immune responses.

In general, the immune system can be divided into innate and adaptive immunity. The innate immune system is the first line of defense against invading agents. The responses are fast and their role is to prevent infections from being established. If the innate immunity fails, the adaptive immune system must respond to clear the established infection and to develop a memory which will prevent from the same infection in the future. Adaptive immunity develops over a life time and adjusts to each infectious encounter.

1.2.1 Innate Immunity

Components of innate immunity are physical barriers such as epithelia and mucous layers on the surfaces of the body, antimicrobial peptides, serum proteins, and innate immune cells including neutrophils, monocytes, macrophages and dendritic cells.

Pattern Recognition Receptors

The first recognition of pathogens by the host occurs when *pathogen associated molecular patterns* (PAMPs) are detected by *pattern recognition receptors* (PRRs). PRRs can be located in the cytosol of host cells, such as *nucleotide-binding oligomerization domain (NOD)-like receptors* (NLRs) and *retinoic acid-inducible gene 1 (RIG-I)-like receptors* (RLRs), or membrane bound such as *Toll-like receptors* (TLRs). Relevant PRR signaling for this thesis is summarized in Figure 3.

In humans, 10 TLRs have been identified and they are either located on the plasma membrane or the endosomal membrane. TLRs are transmembrane proteins that form homo- or heterodimers. Their ectodomains contain leucine-rich repeats responsible for PAMP binding, and the cytosolic *Toll/interleukin-1 receptor* (TIR) domain mediates the intracellular signaling. The TIR domain interacts with TIR-domain containing cytosolic adapters, such as

myeloid differentiation primary response protein 8 (MyD88) and *TIR-domain-containing adapter inducing IFNβ* (TRIF) (51).

All TLRs, apart from TLR3, use MyD88 as an adaptor molecule. MyD88 interacts directly with the TIR-domain of TLRs, or over the sorting adapter *TIR-domain containing adapter protein* (TIRAP) (51, 52). MyD88 recruits *interleukin-1 receptor-associated kinase* (IRAK) family members which have intrinsic serine/threonine kinase activity. Upon stimulation, IRAK4 an IRAK1 autophosphorylate and dissociates from MyD88. They activate *tumor necrosis factor receptor-associated factor 6* (TRAF6) which then activates *transforming growth factor-b-activated protein kinase 1* (TAK1). TAK1 activates the *IκB kinase* (IKK) complex which phosphorylates *inhibitor of nuclear factor (NF)-κB* (IκB) leading to the release of NF-κB from IκB, translocation of NF-κB into the nucleus and transcription of inflammatory genes. TAK1 also activates *mitogen-activated protein kinases* (MAPKs) which lead to the activation of *activating factor-1* (AP-1) and the transcription of inflammatory genes.

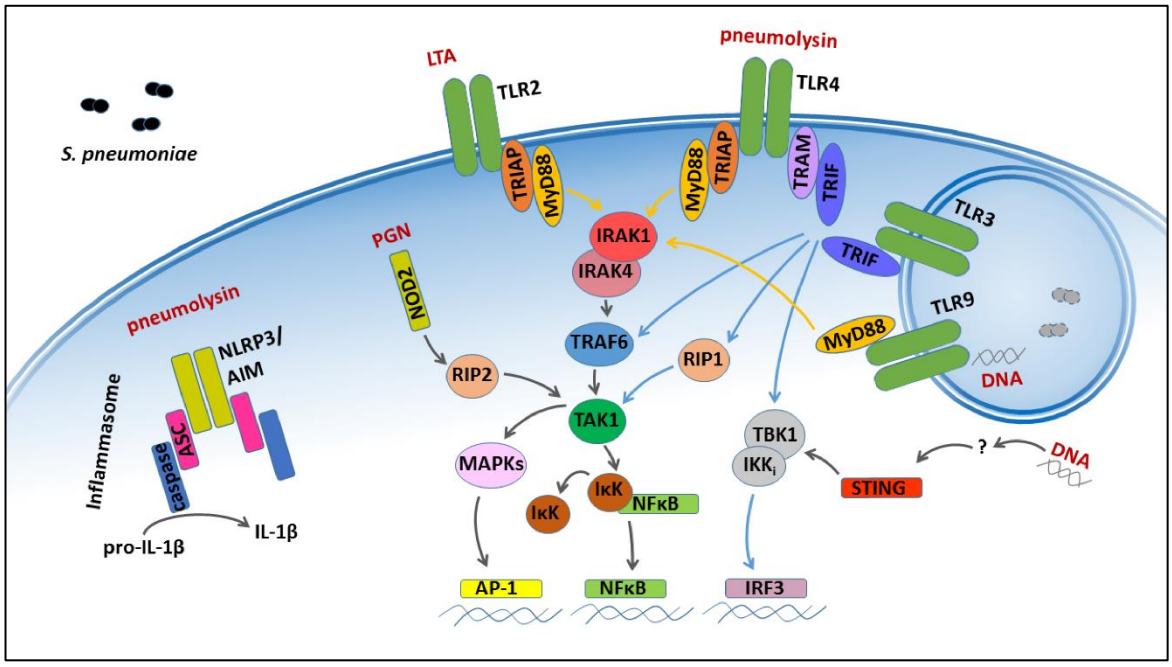

Figure 3 Signaling pathways of selected PRRs and activation of PRRs by *S. pneumoniae*. The TLRs TLR2, TLR4 and TLR9 can be activated by pneumococcal *lipoteichoic acid* (LTA), pneumolysin and DNA, respectively. The activation starts a signaling cascade involving MyD88, IRAK1/4, TRAF6, TAK1 and MAPKs, leading to the activation of the transcription factors AP-1 and NFκB. TLR4 as well as TLR3 activate TRIF which induces transcription of AP-1, NFκB as well as IRF3 regulated genes over the signaling molecules TRAF6, RIP1 or TBK1 and IKK_i. Pneumococcal DNA can also activates an unknown receptor leading to the activation of STING and IRF3 dependent transcription, and *peptidoglycan* (PGN) can activate NOD2 which over RIP2 leads to AP-1 and NFκB activation. The NLRP3 or AIM inflammasome are indirectly activated by pneumolysin leading to the cleavage of pro-IL-1β into IL-1β.

The adapter molecule TRIF is only involved in TLR3- and TLR4-mediated signaling. It directly interacts with TLR3, but requires the sorting adapter *TRIF-related adapter molecule* (TRAM) to bridge the interaction with TLR4. Just as MyD88, TRIF can induce NFκB activation by recruiting TRAF6, but also via activation of *receptor interacting protein* (RIP) 1. Moreover, TRIF interacts with *TANK-binding kinase* (TBK1) which together with IKK_i phosphorylates *interferon regulatory factor* (IRF) 3, leading to the transcription of *interferon* (IFN) β (52).

In summary, the activation of most TLRs leads to the recruitment of MyD88 and the activation of NFκB and AP-1, ultimately leading to the transcription of inflammatory cytokines. Only TLR3 and TLR4 recruit the adapter molecule TRIF, which additionally activates IRF3, leading to the transcription of IFNβ.

The intracellular PRRs of the RLR family are RNA helicases which recognize double stranded viral RNA. RIG-I and *melanoma differentiation-associated protein 5* (MDA-5) belong to this family. They signal over their adapter molecule *mitochondrial antiviral signaling protein* (MAVS), ultimately leading to IRF3 and NFκB activation (53). *Stimulator of IFN genes* (STING) is localized on the endoplasmatic reticulum and mediates signaling in response to sensors of viral DNA leading to IRF3 activation (53).

The NLRs NOD1 and NOD2 are localized in the cytoplasm and recognize bacterial cell wall components. They activate RIP2, leading to the transcription of NFκB and AP-1 regulated genes (53). NLRs such as NLRP3 are the sensors of inflammasome complexes. NLRP3 responds to a variety of stimuli including bacterial cell wall components, extracellular ATP, potassium efflux or crystalline. Due to the large variety in stimuli it is likely that NLRP3 reacts to cellular stress induced by the stimuli, such as potassium efflux, calcium signaling or *reactive oxygen species* (ROS). Activation of NLRPs leads to the recruitment of the adapter *apoptosis-associated speck-like protein containing a caspase activation and recruitment domain* (ASC) and subsequent binding of caspase-1 to ASC. Caspase-1 undergoes cleavage into the active subunits p10 and p20 which cleave the pro-forms of IL-1β and IL-18 into the active forms. Additionally, inflammasome activation can induce a pro-inflammatory type of cell death called pyroptosis (54). Inflammasomes are not only activated by NLRs. They are also activated by *absent in melanoma 2* (AIM2) a DNA binding sensor which also recruits ASC and forms an inflammasome complex (54).

Components of *S. pneumoniae* have been shown to activate several PRRs leading to the secretion of cytokines (Fig. 3). The pneumococcal cell wall component *lipoteichoic acid* (LTA) has been shown to interact with TLR2 (55), TLR9 can be activated by pneumococcal DNA (56), and TLR4 might be activated by the pneumococcal toxin pneumolysin (57-60). Many TLRs are redundant in *in vivo* models and the knockout of TLRs often has only mild or no effects (56, 61, 62). MyD88 in contrast is a central adaptor molecule important for the signaling of most TLRs and a knockout of MyD88 strongly impairs the immune defence against pneumococci (63).

The intracellular receptor NOD2 has been shown to be activated by pneumococcal peptidoglycan and the activation requires presence of the pore forming toxin pneumolysin, probably to promote access of peptidoglycan to the cytosol (64-66). STING can be activated by pneumococcal DNA over an unknown receptor, and similar to NOD2, it requires the presence of pneumolysin for activation (67). Both the NLRP3 and the AIM inflammasome can be activated by pneumococci and this activation also depends on the presence of pneumolysin (68-71).

JAK/STAT signaling

A functional immune system requires communication between the immune cells. This communication happens over direct cell contact, but also by the secretion of cytokines. Cytokines do not only act paracrine, which means that they effect other cell types, but can also act autocrine, affecting the same cell that secreted the cytokine.

A classic example of cytokine signaling is *Janus kinase / Signal Transducers and Activators of Transcription* (JAK/STAT) signaling. JAK/STAT signaling can be activated in response to binding of a cytokine to its cytokine receptor on the cell surface. The binding leads to the dimerization of the receptor, which brings two JAKs, which are bound to the cytosolic part of the receptor, into close contact. The contact leads to their activation and phosphorylation. Subsequently, the JAKs phosphorylate the receptor, creating a STAT binding site. Upon binding to the receptor, STAT is phosphorylated and forms hetero- or homo-dimers. The phosphorylated and dimerized STAT migrates to the nucleus to bind to its binding sequence to regulate the expression of its target genes (72). Four JAKs and seven STATs are found in mammals and they respond to over 50 cytokines and growth hormones (73).

The classical activation of JAK/STAT signaling by type-1 IFNs is shown in Figure 4. Type-1 IFNs bind to the IFN receptor which is a heterodimer composed of IFNAR1 and IFNAR2. Receptor dimerization leads to the activation and phosphorylation of *Tyrosine kinase 2* (TYK2) and JAK1 leading to phosphorylation of STAT1 or STAT2. STAT1 forms a homodimer or a STAT1/STAT2 heterodimer. The heterodimer binds the transcription factor IRF9 to form the *IFN stimulated gene factor 3* (ISGF3) complex which translocates into the nucleus to bind to the *IFN-stimulated response elements* (ISREs). The STAT1 homodimer can directly translocate into the nucleus and binds to *IFN-γ activated site* (GAS) elements (74).

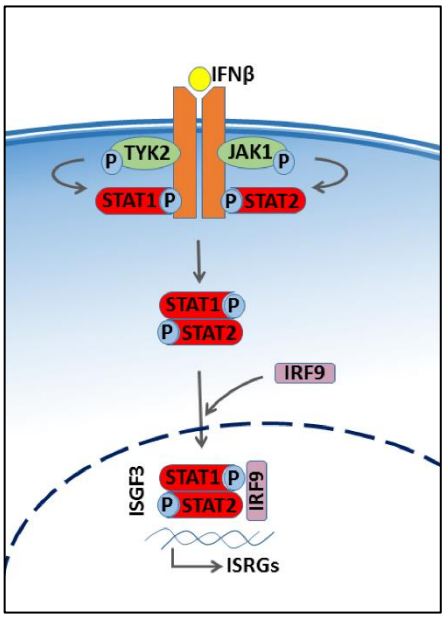

Figure 4 Activation of the JAK/STAT pathway by the type 1 interferon IFNβ. Binding of IFNβ to its receptor induces receptor dimerization and phosphorylation of TYK2 and JAK1, leading to the phosphorylation of STAT1 and STAT2. The STAT proteins dimerize, translocate into the nucleus and together with the transcription factor IRF9, form the ISGF3 complex inducing the transcription of *IFN stimulated response elements* (ISREs).

The complement system

Complement is a class of over 30 serum proteases which are important for the clearance of pathogens. Complement proteins are activated by proteolytic cleavage and bind to the surface of pathogens. Once the first complement proteins are activated, they trigger a hierarchical cascade of proteolytic complement cleavage which rapidly amplifies and results in several outcomes. Complement coats pathogens (a process called opsonization) so that they can be detected and taken up by phagocytes, it forms *membrane attack complexes* (MACs) which lyse pathogens, and it activates inflammation (75). The complement cascade can be activated over three different pathways; the classical, the alternative and the lectin pathway. All pathways lead to the activation of a C3 convertase.

The classical complement pathway is activated when antibodies form a complex with antigens on the pathogen surface. This leads to binding of the C1 complex, formed by the complement proteins C1q, C1r and C1s, to the constant Fc portion of the antibody. The binding activates C1r and C1s which cleave C4 and C2 into C4a, C4b, C2a and C2b. The larger cleavage products assemble to form the C4aC2b C3 convertase, which cleaves C3 into C3b and C3a. C3b binds to the C4aC2b C3 convertase to form the C4aC2bC3b C5-convertase.

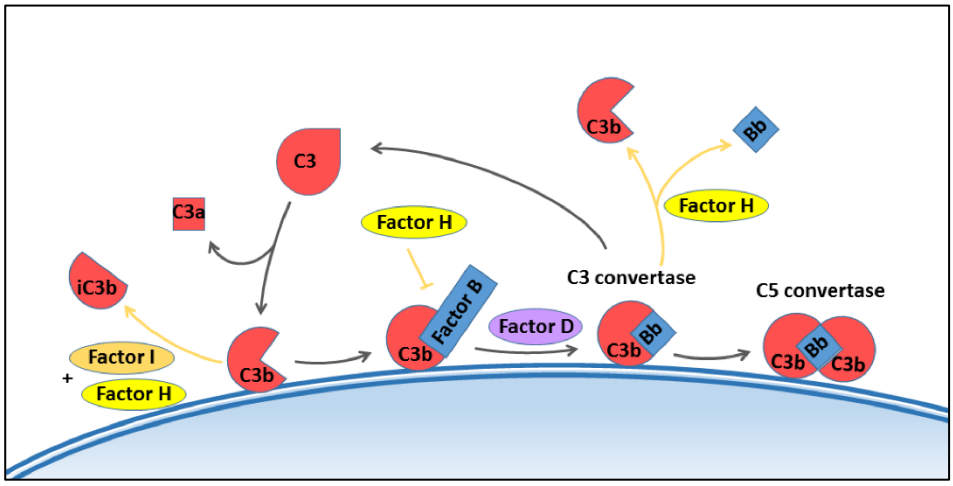

Figure 5 The alternative pathway of complement activation and the inhibitory role of Factor H. After spontaneous hydrolysis of C3, C3b binds to the pathogen surface. C3b binds Factor B which is processed by Factor D to form the C3bBb C3 convertase. The convertase cleaves large amounts of C3 into C3a and C3b to form further C3 convertases and to form the C5 convertase C3bBbC3b. Factor H inhibits the C3b convertase by degrading C3b into iC3b with the help of Factor I. Factor H also inhibits the binding of Factor B to C3b and it promotes the degradation of the C3 convertase.

The lectin pathway is activated when *mannose binding lectin* (MBL) binds to carbohydrate structures on the pathogen. This activates the MBL-associated serine proteases which cleave C2 and C4 leading to the formation of the C4aC2b C3 convertase, and similar to the classical complement pathway, to the activation of the C5 convertase.

The alternative pathway (Fig. 5) is activated by spontaneous hydrolysis of C3 in the serum. When C3 is hydrolyzed into C3a and C3b, the larger product C3b binds to the pathogen surface and together with Factor B and Factor D forms the C3bBb C3 convertase. Cleavage of further C3 proteins leads to the formation of the C3bBbC3b C5 convertase.

The C5-convertase cleaves C5 into C5a and C5b. C5b activation is followed by the activation of further complement proteins (C6-C9) that ultimately lead to the formation of the MAC and lysis of the pathogen.

C3b is a key complement protein, not only because it is part of the C3 convertase, but also because it coats pathogens and is detected by complement receptors that induce phagocytosis and in that way help to clear the infection.

Small products of complement cleavage, such as C3a, C4b and C5a are potent inflammatory proteins that recruit and activate immune cells.

To protect the body's own healthy cells from complement attack, complement activation is tightly regulated. The regulation occurs mainly at the level of the convertases and at the assembly of MACs. Factor H is a protein that contributes to the inhibition of convertases

(Fig. 5). Factor H prevents binding of C3b to Factor B and it is a cofactor for the serum protease Factor I which cleaves C3b into the inactive form iC3b. Factor H also acts as a decay accelerating factor, which means that it accelerates the degradation of the C3bBb C3-convertase (75, 76). Factor H has binding specificity for host cells but pathogens also express Factor H binding proteins on their surface to capture Factor H and to protect themselves from complement killing. Pneumococci express the Factor H binding protein PspC which is studied in this thesis and further described in chapter 1.3.5.

Gram positive bacteria are protected from killing by the MAC due to their thick outer layer of peptidoglycan, and the main effect of complement on these bacteria is to opsonize them for phagocytosis (77). The importance of complement for the prevention of pneumococcal infections is demonstrated by recurrent invasive pneumococcal infections in patients with complement deficiencies (78, 79).

Neutrophils

Neutrophils are constantly generated in the bone marrow and are released into the blood where they constitute 50-70% of the leucocytes. Neutrophils are quickly recruited to the site of infection where they kill pathogens with their granules filled with ROS and antimicrobial proteins. The granules can be releases for extracellular killing of pathogens or fuse with phagolysosomes for intracellular killing. Strongly activated neutrophils can even release their contents including their DNA, histones and the granules to form *neutrophil extracellular traps* (NETs) which immobilize pathogens to limit spread of the infection.

To evade NETs, pneumococci produce endonuclease A, which degrades DNA and releases the bacteria (80). Additionally, the capsule protects pneumococci from getting trapped in NETs (81).

Monocytes

Monocytes are formed in the bone marrow and then enter the blood stream. They constitute 10% of the human leucocyte population in the circulating blood and have diverse functions which support the immune responses. Monocytes help to clear infections by phagocytosis of pathogens, they can present antigen to support adaptive responses and they replenish the reservoir of resident immune cells, such as macrophages and dendritic cells in the dermis and intestine during steady state (82). Alveolar macrophages and dendritic cells are rather maintained by proliferation of local long-lived precursor cells in the lungs (83). During inflammation, however, monocytes also contribute to the reservoir of alveolar macrophages and dendritic cells (82).

Macrophages

Macrophages have high phagocytic activity and express receptors such as lectins, scavenger receptors, Fc-receptors as well as complement receptors to promote uptake of particles.

Macrophages are a highly plastic group of cells and, and develop into different subsets depending on the cytokine environment that they encounter. Traditionally, macrophages have been divided into M1- and M2-macrophages, depending on the *helper T -cell* (T_H-cell) subset that activates them (T-cell subsets are described further in chapter 1.2.2.). M1-macrophages are the classically-activated macrophages that differentiate in response to LPS or the T_H-1 specific cytokine IFNγ. They eliminate intracellular pathogens and produce nitric oxide as well as large amounts of the inflammatory cytokines *interleukin* (IL)-1β and *tumor necrosis factor α* (TNFα) (84, 85). M2-macrophages are alternatively-activated macrophages that differentiate in response to the T_H-2 specific cytokines IL-4 and IL-13. They encapsulate parasites and promote wound healing. M2-macrophages express high levels of *macrophage mannose receptor 1* (MRC-1) and arginase 1 which prevents nitric oxide formation (86, 87). In addition to the T-cell cytokines, *granulocyte-macrophage colony-stimulating factor* (GM-CSF) and *macrophage colony-stimulating factor* (M-CSF) have also been shown to induce the M1- and M2-macrophage like polarization *in vitro* (88). The discovery of new T-cell subtypes led to the description of further macrophage polarizing stimuli, and the division of M2-macrophages into further subtypes (89). However, macrophages encounter a multitude of stimuli in their environment which shape their phenotype and the subtypes rather represent a spectrum in which macrophages can develop.

The ingestion and intracellular killing by macrophages is important for the clearance of pneumococci. Apart from the increased uptake of opsonized pneumococci, macrophages also phagocytose pneumococci via the *macrophage receptor with collagenous structure* (MARCO) (90), *class A macrophage scavenger receptor* (SR-A) (91), *SIGN related-1* (SIGNR1) (92) and MRC-1 (93, 94).

Dendritic cells

Dendritic cells form the link between the innate and adaptive immune responses. Like macrophages, they have phagocytic activity and express lectins, scavenger receptors, Fc-receptors and complement receptors (95). Their main function, however, is not to clear infections by killing of pathogens, but to process the antigen and to present it to T-cells of the adaptive immune system. Dendritic cells are the most efficient *antigen presenting cells* (APCs) of the immune system.

Dendritic cells are rare; they comprise about 1% of the immune cells in most tissues (96). They reside in the mucosal linings of the body and constantly sample antigen, which they present on their surface via the *major histocompatibility complex class II* (MHCII). Upon encounter of a pathogen, dendritic cells become activated, which induces many functional

changes. The activation leads to an increased expression of MHCII on the cell surface which allows for the presentation of large amounts of antigen. Co-stimulatory molecules like *cluster of differentiation* (CD) 80, CD86 and CD40, which are required for a successful interaction with T-cells, are also expressed in high amounts. Depending on the kind of pathogen that the dendritic cell encountered, it secretes specific cytokines. Activated dendritic cells have reduced phagocytic activity and upregulate the expression of the chemokine receptor CCR7 which guides the migration of the cells into the lymph node. In the lymph node dendritic cells meet T-cells to which they present the antigen. Once a dendritic cell interacts with a T-cell expressing a T-cell receptor specific to the presented antigen, this T-cell is activated. Depending on the cytokines that are secreted by the dendritic cell, the T-cell develops into different subtypes (97).

Dendritic cells can be largely divided into three subsets: plasmacytoid dendritic cells, myeloid or conventional dendritic cells and monocyte-derived dendritic cells. They all differ in their capacity to produce cytokines and express different subsets of immune receptors (96). In this thesis, the effect of pneumococcal infections on monocyte-derived DCs has been studied, and they most closely resemble inflammatory myeloid DCs *in vivo* and express most of the TLRs, apart from TLR9 and TLR10 and only low amounts of TLR7 (96, 98).

1.2.2 Adaptive Immunity

T-lymphocytes

T-lymphocytes, also called T-cells, mature in the thymus. Antigen specific T-cells are activated by professional APCs, such as dendritic cells. T-cells are divided into $CD4^+$ and $CD8^+$ T-cells. $CD8^+$ T-cells are also called cytotoxic T-cells and develop in the presence of IL-2. They are activated in response to intracellular antigen such as viral antigen presented on MHCI. In response, they release lytic granules containing perforin and granzyme to induce apoptosis of the infected target cell (99). $CD4^+$ T-cells, also called T_H-cells can develop into several subtypes including T_H-1, T_H-2, T_H-17 and *regulatory T-cells* (Tregs).

T_H-1 cells develop in response to the cytokines IL-12 and IFNγ, and initiate cell-mediated immunity by secreting the cytokines IFNγ and TNFα. The cytokines support intracellular killing by macrophages, which is important for the clearance of intracellular pathogens.

T_H-2 cells develop in response to IL-4 and induce humoral immunity. They produce the cytokines IL-4, IL-5 and IL-13, and activate B-cells to undergo affinity maturation and isotype switching. This process is required for the production *immunoglobulin* (Ig) G, IgA and IgE antibodies of high affinity to fight extracellular pathogens.

T_H-17 cells develop in response to IL-6, IL-23, and TGFβ. IL-23 is a cytokine similar to IL-12. Both cytokines contain the subunit p35, which combined with p40 forms IL-12 and with p19 forms IL-23. T_H-17 cells are pro-inflammatory and produce IL-17, a cytokine involved in the recruitment of neutrophils.

Tregs produce the anti-inflammatory cytokines IL-10 and *Transforming growth factor β* (TGF-β) and regulate cell-mediated immunity as well as B-cell responses (99).

Several T-cell subsets are important in clearing colonization and infections with *S. pneumoniae*. In humans it has been shown that T_H-1 cells disappear from the blood during pneumococcal infections, which is thought to be due to their migration and help in the tissue (100). IL-12, the cytokine that drives the development of T_H-1 cells, seems to be important for the immune response towards pneumococci, since a patient with severe IL-12 deficiency suffered from recurrent pneumococcal infections (101). Additionally, IFNγ which is produced by T_H-1 cells, has been shown to be protective in *in vivo* mouse models (102, 103). In summary, a T_H-1 phenotype seems to be beneficial to clear pneumococcal infections.

It has been reported that T_H-17 cells are involved in mediating an antibody independent protective immunity to pneumococci (104) and that they are important for the clearing of pneumococcal carriage in naive mice (105). This protection is mediated by the recruitment of phagocytes to the tissue which clear the colonizing bacteria (105). A human colonization model showed that carriage with pneumococci significantly enhanced the numbers of IL-$17A^+$ and $CD4^+$ memory cells in the blood and lungs (106). Studies of mucosal tissue from children and adults have shown that pneumococcus-specific T_H-1 and T_H-17 cells sequester with increasing age (107, 108).

Knowledge about the role of Tregs during pneumococcal infections is just emerging within the last years. Comparison of Balb/c mice, which are more resistant to pneumococcal infections, to CBA/ca mice, which are more susceptible to pneumococcal infections, revealed a higher TGF-β production and a higher number of Tregs in the lungs of Balb/c mice. Adoptive transfer experiments confirmed that Tregs have a protective role during pneumococcal infections in a murine model (109). Nevertheless, studies of human nasal associated lymphoid tissue indicate that pneumococcal carriage coincides with low levels of T_H-17 and high levels of Tregs (108, 110), implicating a negative effect of Tregs in the clearing of colonization.

Although the role of the different T-cell subsets during pneumococcal infections is not fully understood, emerging data implicates an importance of T_H-1 and T_H-17 for the prevention of colonization and disease.

B-lymphocytes

B-lymphocytes, also called B-cells, are the cells of the immune system that produce antibodies. In the context of an infection, B-cells take up antigen and present it on MHCII. T_H-cells with specificity for this antigen can activate the B-cell to undergo affinity maturation and isotype switching. This leads to the formation of long lived plasma cells producing antibodies of type IgG, IgE and IgA, and to the differentiation of memory B-cells.

Alternatively, B-cells can be activated in a T-cell independent manner. This happens in response to pure polysaccharides of the pneumococcal capsule, such as in the PPV23 vaccine. These anionic polysaccharides are not able to bind to MHCII, and therefore T-cells cannot be activated by dendritic cells and B-cells cannot present the antigen. Instead, polysaccharides activate B-cells by crosslinking the B-cell receptors, but without T-cell help they do not undergo memory B-cell differentiation, affinity maturation and isotype switching. The B-cells develop into short lived plasma cells that produce antibodies mainly of the type IgM. The produced antibodies have low affinity and do not provide long lasting immunity. Children under the age of 2 years are not able to induce this T-cell independent B-cell activation because their B-cells are not fully developed (111).

In conjugated vaccines like PCV7, the polysaccharides are bound to a carrier protein. This protein can be presented on MHCII and initiates T-cell dependent B-cell activation as during a normal infection process. This induces affinity maturation, isotype switching and differentiation into long lasting memory cells (111).

IgA is an antibody class important for mucosal immunity. Nevertheless, its contribution to the prevention of pneumococcal infections is not clear. Selective IgA deficiency is the most common immunodeficiency in Western countries and 1/600 individuals is affected. Although the affected individuals lack the mucosal IgA antibodies, they rarely have an increased risk for infections. IgG can be divided into 4 subclasses (IgG_1, IgG_2, IgG_3 and IgG_4) and IgG_2 antibodies are formed towards capsular polysaccharides. A deficiency in IgG_2 is associated with recurrent respiratory tract infections (112).

1.2.3 Immunomodulation by Vitamin D

Vitamin D is produced in the skin upon exposure to sunlight. The *ultraviolet* (UV) B radiation of the sun leads to photolytic cleavage of 7-dehydrocholesterol into pre-vitamin D_3 which by thermal isomerization becomes vitamin D_3 (cholecalciferol). Apart from endogenous vitamin D_3 production in the skin, vitamin D_3 can also be adsorbed from food sources in the intestine. Activation of vitamin D_3 requires two hydroxylation steps. First vitamin D is transported to the liver where it is hydroxylated by the 25-hydroxylase to $25(OH)D_3$ (calcidiol). $25(OH)D_3$ is the most common circulating form of vitamin D in the

blood and is used to determine the vitamin D status of individuals. $25(OH)D_3$ is further hydroxylated to $1,25(OH)_2D_3$ (calcitriol) by the 1α-hydroxylase (Cyp27B1) in the kidneys and in other tissues. $1,25(OH)_2D_3$ is the active form of vitamin D and can bind to the *vitamin D receptor* (VDR) which is present in nearly all vertebrate cell types (113). The VDR together with the *retinoid X receptor* (RXR) binds to the *vitamin D response elements* (VDREs) and regulates the transcription of over 200 genes (114).

Vitamin D is important for calcium absorption from the intestine and for mineralization of the bones. The classic disease associated with vitamin D deficiency is rickets, marked by defects in calcium metabolism leading to deformations and fractures of bones. However, an immunomodulatory role of vitamin D on the innate and adaptive immune responses also becomes increasingly clear.

Immunomodulatory effects of vitamin D have been described for many cell types. Vitamin D supports innate immune responses by inducing the production of antimicrobial peptides, such as cathelicidin (LL-37) and human β defensins, and enhances the antibacterial activity of monocytes and macrophages (115, 116). In the presence of vitamin D, adaptive immune responses are dampened and monocytes differentiate into dendritic cells with an inhibitory phenotype. Maturation, IL-12 production and T-cell activation is strongly reduced in these dendritic cells, while they secrete increased amounts of IL-10 (117). Vitamin D inhibits T-cell proliferation and modulates the T-cell phenotype; it reduces T_H-1, T_H-2 and T_H-17 responses whereas it supports the development of Tregs (118, 119).

A positive effect of vitamin D on *respiratory tract infections* (RTIs) was suspected when children suffering from rickets also were found to have an increased risk for RTIs (120). The prototypical example of a connection between vitamin D and infections is tuberculosis. A correlation between low vitamin D levels and tuberculosis has long been suspected and this connection was recently confirmed in two larger observational studies (121, 122).

Likewise, an association between low serum levels and an increased risk for RTIs has been found in observational several studies (123, 124). However, a direct causality has not been proven and *randomized placebo controlled trails* (RCTs) evaluating the effect of vitamin D supplementation on the prevention of RTIs were not conclusive. The two most recent systemic reviews and meta-analyses found large heterogeneity between the RCTs and the role of vitamin D in prevention of RTIs is still unclear (125, 126).

1.3 PNEUMOCOCCAL VIRULENCE FACTORS AND THE HOST

During pneumococcal colonization and invasive disease, the bacteria are in a constant interplay with the host. While the immune system detects pneumococci with the help of PRRs, antibodies and the complement system, pneumococci have developed strategies to evade and modulate the immune responses to their benefit. The pneumococcal cell wall with the anti-phagocytic capsule and the virulence factors autolysin, pneumolysin and PspC will be discussed in this chapter.

1.3.1 The Cell Wall

Pneumococci are surrounded by a Gram-positive cell wall, which consists of a thick layer of peptidoglycan and teichoic acids (Fig. 6). Peptidoglycan is a multilayered structure of long glycan chains composed of *N-acetylglucosamine* (GlcNAc) and *N-acetlymuramic acid* (MurNAc). The glycan layers are cross-linked with peptide chains. *Teichoic acids* (TAs) are highly conserved in pneumococci and they consist of repeating units of sugars. They can be divided into *lipoteichoic acids* (LTAs) which are linked to the cytoplasmic membrane and *wall teichoic acids* (WTA) which are attached to peptidoglycan. TAs are decorated with phosphocholine residues, which play an important role as anchors for the choline binding surface proteins of pneumococci (127). The cell wall also contains surface proteins with a LPxTG motif, that are covalently linked to the peptidoglycan by sortase catalyzed transpeptidase reactions, and lipoproteins that are attached to the cytoplasmic membrane.

The cell wall is vital to keep the shape of the bacteria and to protect them from bursting. However, it also contains components that are detected by the immune system and cause an inflammatory response. Peptidoglycan can be released into the cytosol when the endosome is lysed by the pneumococcal toxin pneumolysin, leading to activation of NOD2 (64-66). LTAs have been reported to activate TLR2 (55) although more recent studies show that the role of LTA in TLR2 activation is limited and that the activation mainly results from lipoproteins found in the LTA preparations (128). To avoid the detection by the immune system, the pneumococcal cell wall is surrounded by a polysaccharide capsule.

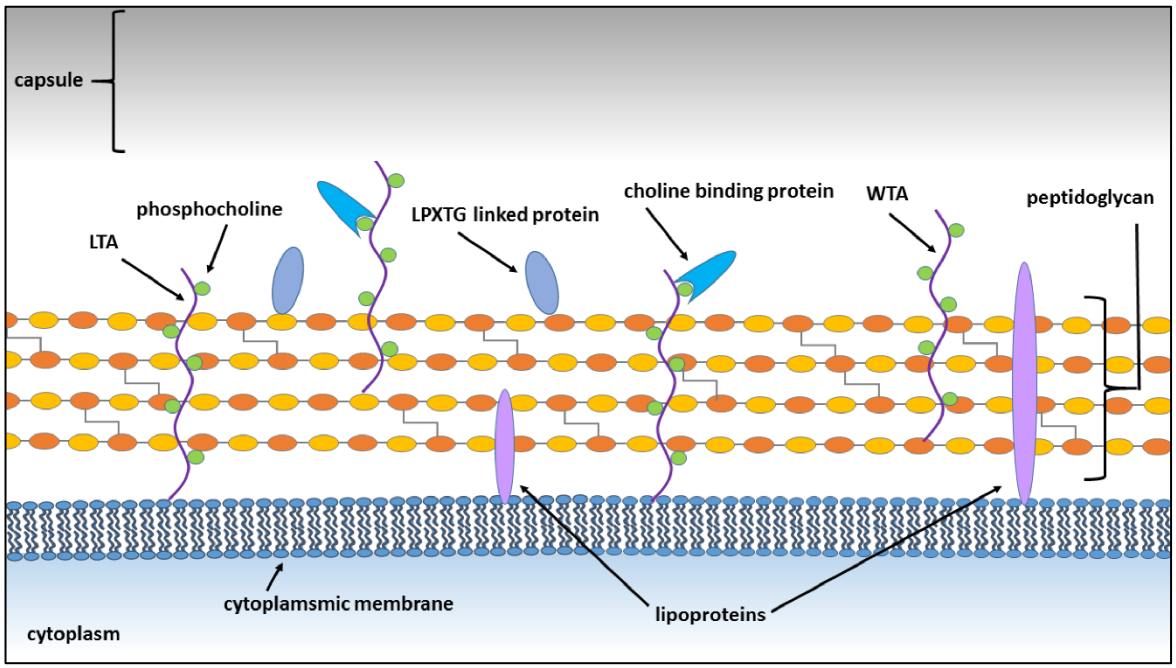

Figure 6. The pneumococcal cell wall. The cell wall consists of a thick layer of peptidoglycan covering the cytoplasmic membrane as well as *lipoteichoic acids* (LTA) and *wall teichoic acids* (WTA). The teichoic acids are decorated with phosphocholine residues. Proteins are attached to the lipid layer (lipoproteins), to phosphocholine (choline binding proteins) or to peptidoglycan (LPxTG linked proteins). The cell wall is surrounded by the capsule.

1.3.2 The Capsule

The pneumococcal cell wall is surrounded by a polysaccharide capsule which is highly diverse in saccharide composition (129). Due to this large variation in the capsule, protective antibodies against pneumococci are specific to only one serotype or one serogroup and do usually not protect from infections with other serogroups. The capsule protects the bacteria from opsonization with complement, and is a major factor determining the extent of complement deposition (130), although the genetic background of pneumococci also contributes (130, 131). A consequence of the reduced opsonization but also of the predominantly negative charge of the capsule is the decreased phagocytosis of encapsulated pneumococci (129, 130). Additionally, the capsule prevents pneumococci from getting trapped in NETs released by neutrophils (81) or the mucous in the lungs (132).

The capsule is the major virulence factor of pneumococci and while non-encapsulated *S. pneumoniae* strains compose 9-13% of the carriage isolates, they are rarely associated with invasive disease (133). While the capsule is an important virulence factor and protects bacteria from phagocytic killing in the blood stream, it might also hinder the adhesion during colonization and infection of the lungs. Phase variation is a phenomenon which might help

the bacteria to overcome this dilemma. It has been shown that pneumococci spontaneously can switch between a transparent and an opaque phenotype of which the former is able to colonize the nasopharynx (134) and the latter is virulent in an invasive model (135). Interestingly, the transparent phenotype is associated with a reduced capsule production (135), but the phenotypic changes are also affecting other pneumococcal virulence factors (136). Visualization of pneumococci together with epithelial cells revealed that the bacteria in close contact with the cells produce reduced amounts of capsule compared to bacteria that do not have contact with cells (137), supporting that pneumococci might decrease capsule production for close interactions with epithelia.

1.3.3 Autolysin

Pneumococcal cultures in stationary phase undergo a characteristic lysis *in vitro* and the protein responsible for this is the major autolysin LytA. LytA is a choline binding protein with amidase activity. The amidase acts on peptidoglycan and cleaves the lactyl-amide bond between MurNAc in the glycan strand and the stem peptide of the peptide chain (138), which destabilizes the bacterial cell wall and causes autolysis. LytA also mediates sensitivity to cell wall-acting antibiotics, such as penicillin G or vancomycin (139, 140). The regulation and the molecular mechanism of LytA activity is not fully understood, but it is known that the protein is primarily localized in the cytoplasm during early exponential growth and is released into the medium during stationary and lytic phase. It binds to the bacterial surface and localizes to the equatorial division site, where the nascent peptidoglycan is synthesized (140). LytA is activated by the disruption of cell wall synthesis and requires long glycan chains as substrates. The present knowledge points towards a regulation of LytA activity by substrate recognition and that it might specifically recognize nascent peptidoglycan at the equatorial plain during growth inhibition. (140, 141).

LytA is required for virulence in *in vivo* models of meningitis (142), intra peritoneal infection (143), intravenous infection (144) and pneumonia (145) but the function of the autolysin during pathogenesis is not fully understood. The virulence for LytA can to a large extend be explained by the release of the toxin pneumolysin during autolysis (142, 143, 146) but also pneumolysin independent immunomodulation by LytA has been reported (147). Additionally, LytA might contribute to lysis of pneumococci during competence and increase transformation of competent pneumococci with the released DNA (148). Recently, a role for LytA in capsule shedding in response to the antimicrobial peptide LL-37 has been described (149).

1.3.4 Pneumolysin

Pneumolysin is a 53 kDa cholesterol binding cytolysin expressed by virtually all invasive isolates of pneumococci (150). At high concentration, the toxin forms pores in cholesterol containing cell membranes and induces lysis of host cells. However, cytolytic activity of pneumolysin is not required to cause disease, since a non-hemolytic version of pneumolysin can be found in serotype 1 strains, which are associated with pneumococcal disease outbreaks (151, 152).

The crystal structure of pneumolysin has recently been solved and shows that the protein is build up in 4 domains. Domain 4 on the C-terminal part of the protein interacts with cholesterol in plasma membranes, but it can also act as a lectin and bind mannose or the blood type sugar LewisX (153-155). It is the current understanding that pneumolysin monomers bind to the cell membrane and form multimers of 30-50 molecules to assemble a pre-pore. Upon pre-pore assembly, the multimer undergoes a large conformational change, leading to the perturbation of the membrane by domain 4 and the formation of a pore with a 320-430 Å diameter (156). No active transport mechanism for pneumolysin has been identified and it is therefore believed that the toxin is released during autolysis of the bacteria. Extracellular pneumolysin mainly localizes to the pneumococcal cell wall but is also found in the culture supernatant of pneumococci (157). Pneumolysin can activate the classical complement pathway (158) and this is believed to be due to structural similarity of domain 4 to the Fc-portion of antibodies (159).

At high concentrations, pneumolysin induces cell death by pore formation and slows down ciliary beating of respiratory epithelium (160, 161). At sublytic concentrations pneumolysin can form micropores, and a range of modulating effects on host responses have been identified. It has been shown that pneumolysin can rearrange the cytoskeleton of neuroblastoma cells and astrocytes. It directly interacts through lipid layers with actin and it can activate the GTPases Rho1 and RacA which modulate the actin cytoskeleton. This leads to the formation of stress fibers, lamelopodia and filopodia (162, 163). Pneumolysin also induces microtubule bundling at sublytic concentrations, but the mechanisms behind this are not understood. The toxin does not directly in interact with microtubule and the mechanisms leading to the bundling might be multifactorial (164).

A well-documented function of pneumolysin is the induction of pro-inflammatory cytokines. Pneumolysin can activate the NLRP3 and AIM2 inflammasomes leading to the production of IL-1β and IL-17 (68, 70, 71). The activation requires the presence of cytolytic pneumolysin and is not induced by serotype 1 and 8 (165). Furthermore, several reports show an activation of TLR4 by pneumolysin leading to the secretion of cytokines (57-60) whereas other studies report TLR4 independent cytokine secretion (68, 166, 167). Recently it has also been shown that pneumolysin has the capacity to permeabilize endolysosomal membranes, leading to the release of peptidoglycan into the cytosol which might activate NOD receptors (66).

Anti-inflammatory or inhibitory functions of pneumolysin are less frequently described. In the 1980's it was reported that pneumolysin inhibited the activation, proliferation, and antibody production of lymphocytes (168), as well as the respiratory burst and antimicrobial activities in monocytes and neutrophils (169, 170). It remained unclear in these studies to which extend the inhibition was due to cytotoxic effects of pneumolysin. Littmann et al. (171) showed that dendritic cell activation, maturation and cytokine secretion is inhibited by pneumolysin. The inhibition could largely but not fully be explained by the induction of apoptosis and cell death in dendritic cells.

1.3.5 Pneumococcal surface protein C

PspC is an important virulence factor of pneumococci. It is a highly polymorphic protein and based on sequence homology, 11 major groups of PspC have been identified. PspCs of group 1-6 bind to the bacterial cell wall via a choline binding domain, and group 7-11 bind to the cell wall via a LPxTG motif (172). Some pneumococci, including clinical isolates of serotype 6B belonging to *clonal complex* (CC) 138, express two PspC proteins of which one has a choline binding domain and one a LPxTG motif (172, 173).

Functionally, PspC is very diverse but mainly mediates immune evasion by binding to Factor H (174) and preventing C3b deposition (175). Additionally, PspC contributes to adhesion to host tissue. PspC exerts adhesive functions by interacting with the *secretory component* (SC) of secretory IgA and the *poly Ig receptor* (pIgR) (176). The interaction with SC of pIgR has been shown to mediate invasion of (177, 178) and translocation through epithelia cells (179). PspC also mediates adherence by interacting with extracellular matrix proteins such as vitronectin (180) and human thrombospondin-1 (181). The binding of Factor H to PspC also supports adhesion to host cells (182).

Due to the multiple functions and allelic variations of PspC, the protein has also been called *choline binding protein A* (CbpA), *Factor H inhibitor of complement* (Hic) and *Streptococcus pneumoniae secretory IgA binding protein* (SpsA).

PspC contributes to colonization, pneumonia and bacteremia in murine models (183, 184). However interestingly, PspC interacts specifically with human and not murine secretory IgA (179, 185), SCpIgR (185) and Factor H (186), offering a possible explanation for the species specificity of pneumococci to infect humans.

1.3.6 Pathogenesis of Influenza Pneumococcal Coinfections

Infection with influenza virus predisposes the host for a superinfection with *S. pneumoniae*. The mechanisms underlying the increased susceptibility and more severe infections are not fully understood but experimental evidence, mainly from murine models, suggests a contribution of multiple factors.

Influenza induces changes in the lung environment which promote pneumococcal infections, such as damage to the lung epithelia and mucosa. The viral neuraminidase has been shown to cleave off sialic acids from the lung, exposing receptors required for pneumococcal adherence (187) and the released sialic acids also provide a nutrient source for pneumococcal growth (188). Additionally, influenza infection desensitizes TLRs in alveolar macrophages, which lasts for several weeks after the infection (189). At the same time coinfections induce a cytokine storm in the lungs and the increased inflammation might support the bacterial infection (190, 191).

The viral infection also affects the amount and function of immune cells in the lungs. It has been shown that the numbers of neutrophils in the lungs increase and the numbers of alveolar macrophages decrease seven days post influenza infection. This correlates with the peak of susceptibility for pneumococcal infections. Nevertheless, it is not clear to which extent the neutrophil influx affects the bacterial infection (33). It is clear however, that alveolar macrophages are crucial to prevent and clear pneumococcal infections and the depletion of macrophages during influenza infection has been shown to account at least partially for the enhanced pneumococcal virulence (192). Additional to the reduced number of macrophages in the lungs, macrophage function is impaired. IFNγ levels are increased during influenza pneumococcal coinfections and the cytokine reduces the expression of MARCO, a scavenger receptor involved in phagocytosis of pneumococci, on macrophages which leads to impaired clearance and more severe disease outcome (193). Type I interferons are also induced by influenza and they have been shown to compromise T-cell function which leads to reduced IL-17 production and increased virulence of pneumococci (194). The role of dendritic cells during coinfections is not well studied. It has been shown that dendritic cell numbers in the lungs are not affected during coinfections (195). A cytokine boost in influenza primed human dendritic cells infected with pneumococci has been observed *in vitro* (196, 197). The altered cytokine secretion in dendritic cells might contribute to the cytokine storm in the lung and the impaired macrophage and T-cell functions.

2 AIMS

The general aim of this thesis was to explore the interactions between *S. pneumoniae* and the immune system. A focus was put on the innate immune responses of macrophages and dendritic cells. The studies should contribute to the knowledge about pneumococcal factors that activate or evade immune responses and about possible modulations of the immune responses to pneumococci to benefit the host.

2.1 SPECIFIC AIMS

Paper I

To investigate pneumococcal components and host receptors required for induction of cytokine secretion by dendritic cells. A mechanism underlying the increased cytokine responses of dendritic cells in coinfection with influenza A virus and *S. pneumoniae* is described.

Paper II

To describe the differential effects of the pneumococcal toxin pneumolysin on immune cells and to investigate mechanisms behind the inhibitory effects of pneumolysin on dendritic cells.

Paper III

To characterize two variants of the pneumococcal protein PspC expressed in B6 clinical isolates regarding their localization on the bacterial surface as well as their role in complement evasion and adhesion to host cells.

Paper IV

To explore the immunomodulatory effects of vitamin D on dendritic cells and T-cells in the context of pneumococcal infection.

3 METHODOLOGICAL CONSIDERATIONS

Bacteria:

In this thesis, pneumococcal strains of serotype 2, 4 and 6B have been studied.

TIGR4 (T4) an invasive serotype 4 isolate from a Norwegian patient (198) was used in paper I, II, III and IV. The pneumococcal capsule prevents efficient uptake of pneumococci in *in vitro* assays. It has previously been shown that phagocytosis rates can be increased by opsonisation of the bacteria or by using an unencapsulated mutant (171). Therefore, we used an isogenic unencapsulated mutant of serotype 4 (T4R) (199), in paper I, II and IV, as well as opsonized T4 in paper I.

Serotype 6B isolates belonging to CC138 were used in paper III. CC138 is common in colonization and associated with high mortality rates (173, 200). The isolates BHN191 and BHN418 of CC138 have previously been shown to express two closely linked copies of the *pspC* allele, which differ in their domains for cell wall attachment. *pspC1* encodes a choline binding domain, and *pspC2* encodes a LPxTG motif. BHN191 is a nasopharyngeal isolate from a healthy child, and BHN418 is isolated from blood of a meningitis patient. We used the isolates to study the function of the two PspC proteins in paper III. Mutants of the *pspC* genes were created by fusion PCR mutagenesis. The serotype 2 strain D39 and T4 were also used in this study because they only express one copy of the *pspC* allele.

Virus:

Severe pneumococcal infections occur in close temporal proximity after infections with influenza virus. Our group has previously studied the effects of *S. pneumoniae* and *influenza A virus* (IAV) coinfections on dendritic cells (196) and in paper I we follow up on this study. We used the X31 strain of IAV propagated in *Madine-Darby canine kidney* (MDCK) cells. Dendritic cells were stimulated for 2 hours with IAV and subsequently infected with T4R.

Immunostimulation:

Paper I, II and IV investigate the immune response to pneumococci and pneumococcal components. The pneumococcal component used in paper I was total RNA isolated from T4R. Pneumococcal peptidoglycan was used as a stimulant in paper IV.

Substances to stimulate specific PRRs were used in paper I, II and IV (Table 2). *Polyinosinic-polycytidylic acid* (Poly I:C) is a synthetic double stranded-RNA analog which activates TLR3. *Lipopolysaccharide* (LPS) is a component of the Gram negative cell wall and a strong activator of TLR4. R848, also called Resquimod, is a guanosine derivate which activates

TLR7 and TLR8. *Muramyl dipeptide* (MDP) is a small component of peptidoglycan, known to stimulate NOD2.

Table 2 TLR agonists used in this thesis.

Substance	PRR	Included in
Poly I:C	TLR3	Paper I
LPS	TLR4	Paper I, II and IV
R848	TLR7 and TLR8	Paper I
MDP	NOD2	Paper IV

Cells:

This thesis investigates the effects of pneumococci and pneumococcal components on human immune cells. While much research has been performed on the effect of pneumococci on murine immune cells, we used to a large extent immune cells derived from human primary monocytes. Mice are not a natural host for pneumococci and the human and murine immune system differ in aspects that were important for the studies of this thesis. Cytokine responses to pneumococci, which were studied in paper I and II and IV, differ significantly between murine dendritic cells and human dendritic cells (171). The pneumococcal protein PspC, which is studied in paper III is known to interact with human Factor H but does not bind Factor H from other species (186). Immunomodulation by vitamin D, studied in paper IV, differs in the human and the murine system, especially the expression of antimicrobial peptides is differentially regulated (201).

The following cell types were used in this study:

Human monocytes

Human monocytes were isolated from buffy coats from healthy donors, provided by Karolinska University Hospital. $CD14^+$ monocytes were isolated by negative selection with the RosetteSep human monocyte enrichment cocktail (StemCell Technologies) and a gradient centrifugation with Ficoll-Plaque plus (GE healthcare). The cells were used for the differentiation of dendritic cells and macrophages in paper I and II. In paper IV, *peripheral blood mononuclear cells* (PBMCs) were isolated by gradient centrifugation with Ficoll-Plaque plus (GE healthcare), and monocytes were allowed to adhere to culture flasks for 2 hours. Non-adherent cells were carefully washed off.

Human monocyte derived dendritic cells

Human monocyte derived dendritic cells were used in paper I, II and IV. They were differentiated from human monocytes in the presence of IL-4 and GM-CSF (Peprotech) for 6 days. The cells were over 90% positive for CD1a and CD11c.

Human monocyte derived macrophages

Human monocyte derived macrophages were used in paper II. They were differentiated from human monocytes for 6 days in the presence of GM-CSF or M-CSF (Peprotech) into M1-like macrophages or M2-like macrophages, respectively.

Human $CD4^+$ naïve and memory T-cells

Human $CD4^+$ naïve and memory T-cells were cocultured with stimulated autologous dendritic cells in paper IV. The T-cells were isolated with the EasySep human memory $CD4^+$ T-cell enrichment kit, or with the EasySep human naïve $CD4^+$ T-cell enrichment kit (StemCell Technologies) from non-adherent PBMCs.

THP-1 derived macrophages

THP-1 cells are a monocytic human cell line derived from an acute monocytic leukemia patient (American Type Culture Collection [ATCC], Manassas, VA). The cells were differentiated for 48 h with *phorbol myristate acetate* (PMA) (Sigma) into macrophage-like cells which were used in paper II and III.

A549 cells

A549 cells are alveolar basal epithelia cells derived from a human lung adenocarcinoma (American Type Culture Collection [ATCC], Manassas, VA). The cells are used in paper III to study the adhesion of pneumococci to host cells.

HEK293 cells

Human embryonic kidney 293 (HEK293) cell originate from healthy fetal kidney cells transformed with adenovirus DNA. HEK293 cells stably transfected with plasmids expressing either TLR4, MD2, CD14 and luciferase under the regulation of the NFκB promoter, or TLR3 and luciferase under the ELAM promoter were used in paper I. The cells were transfected with pneumococcal RNA and luciferase activity was measured to study the activation of the receptors.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Toll-like receptor 3/TRIF-dependent IL-12p70 secretion mediated by *Streptococcus pneumoniae* RNA and its priming by influenza A virus coinfection in human dendritic cells

In paper I we investigate the recognition of *S. pneumoniae* by dendritic cells, which leads to the secretion of the cytokine IL-12p70. Additionally, we studied the effect of prior *influenza A virus* (IAV) infection on the cytokine secretion.

When we silenced the adapter molecule TRIF with *small interfering RNA* (siRNA) in dendritic cells, we observed a marked decrease in IL-12p70 secretion in response to pneumococci. The only receptors known to recruit the adapter TRIF are TLR3 and TLR4 (202, 203). Due to the previously reported activation of TLR4 by the pneumococcal toxin pneumolysin (57-60) we hypothesized that TRIF is mediating signals from TLR4 leading to the secretion of IL-12p70. However, silencing of TLR4 with siRNA revealed that IL-12p70 secretion is independent of TLR4. The activation of TLR4 by pneumococci is still debated (68, 166, 167) and differences might stem from contaminants in the pneumolysin preparations as well as differences in the model system. Our data indicate that pneumococci induce IL-12p70 secretion in dendritic cells in a TLR4 independent manner.

Due to the lack of TLR4 activation we investigated the role of TLR3, a receptor for *double stranded RNA* (dsRNA) previously not shown to be activated by pneumococci. We found that silencing of TLR3 with siRNA significantly reduced IL-12p70 secretion in dendritic cells, a result which we could confirm with the use of a chemical TLR3/dsRNA complex inhibitor. The activation of an endosomal receptor like TLR3 is further supported by the requirement of bacterial uptake by dendritic cells for IL-12p70 secretion.

The results were surprising, since TLR3 is known to be activated by viral dsRNA and bacteria produce only *single stranded RNA* (ssRNA). However, secondary RNA structures such as in *ribosomal RNA* (rRNA) or *transfer RNA* (tRNA) might be able to activate TLR3. The role of bacterial RNA in the activation of PRRs has just emerged within the last years. Several studies have shown that RNA isolated from bacteria can activate cells in a TLR3 dependent (204-207) or independent manner (208-214). RNA might be an important signal of bacterial viability to the immune system (212). Pneumococcal RNA as a signal for TLR3 had not been investigated previously.

To study the role of pneumococcal RNA in TLR3 activation, we transfected total RNA into dendritic cells as well as HEK293 cells expressing TLR3. Pneumococcal RNA was sufficient to activate the HEK293 cells and to induce IL-12p70 secretion in dendritic cells in a TLR3 dependent manner. Additionally, we stimulated dendritic cells with UV-killed pneumococci

which were pretreated with RNAses, and confirmed the importance of RNA as a pneumococcal stimulus in dendritic cells.

Our group had previously observed that IAV infection could prime dendritic cells to secrete increased amounts of the cytokines IL-6 and IL-12p70 during subsequent pneumococcal infection. The priming of dendritic cells was mediated by a soluble factor and IFNα was a sufficient stimulus to prime the cells (196). Since viral infection as well as type I interferons can increase the expression of TLR3 (215, 216), we investigated TLR3 expression as a possible mechanism underlying the increased cytokine secretion in pneumococcal IAV coinfection. TLR3 expression was enhanced by IAV and, to a lesser extent, by IFNα. Additionally, the increased IL-12p70 secretion in pneumococcal IAV coinfection was inhibited by the TLR3/dsRNA complex inhibitor. The data indicates that IAV upregulates TLR3, probably by soluble factors such as IFNα, and the increase in receptors contributes to the enhanced IL-12p70 secretion in pneumococcal IAV coinfections.

In summary, we found that dendritic cells sense pneumococcal RNA via the receptor TLR3. TLR3 recruits TRIF, which ultimately leads to the expression and secretion of IL-12. This signaling in dendritic cells might be of particular importance during pneumococcal IAV coinfections, due to the upregulation of TLR3 by the virus, leading to an increased cytokine response to pneumococci.

IL-12 is an important part of the immune responses against pneumococcal infections. It induces the differentiation of T-cells into T_H-1 cells which produce IFNγ and support clearance of pneumococcal infections (102, 103). IL-12 deficient mice have decreased survival in a pneumococcal pneumonia model (103, 217) and a patient with a severe IL-12 deficiency suffered from recurrent pneumococcal infections (101). However, uncontrolled cytokine production as in coinfections with IAV and *S. pneumoniae* can damage the lungs and negatively affect disease outcome (191). Enhanced IFNγ in IAV pneumococcal coinfections impairs clearance by macrophages and has detrimental effects on survival in murine models (193). Concluding, tightly regulated secretion of cytokines is important for a positive disease outcome, and TLR3 activation by pneumococcal RNA in dendritic cells might contribute to clearance of pneumococci but also to pathogenesis.

4.2 PAPER II

Pneumococcal toxin pneumolysin mediates cell type specific inhibition of cytokine secretion

The cytolytic toxin pneumolysin is an important virulence factor required for invasive pneumococcal disease in murine models (143, 144). The proinflammatory effects of pneumolysin are well established (218), although it is not clear whether pneumolysin mediates the proinflammatory effects via the receptor TLR4 (57-60) or in a TLR4 independent manner (68, 166, 167). Inhibitory effects of pneumolysin on immune cells have been reported in the 1980's by the Paton group which described an inhibition of the functions of human neutrophils, lymphocytes and monocytes (168-170). However, it is unclear whether these effects were a consequence of pneumolysin induced cell death. Our group reported that pneumolysin expression by pneumococci inhibits dendritic cell functions, which largely but not fully correlated with pneumolysin induced cell death (171).

In paper II we compared the effects of pneumolysin on different cell types. We infected dendritic cells, M1-like macrophages differentiated with GM-CSF, M2-like macrophages differentiated with M-CSF and THP-1 derived macrophages with a low dose of pneumolysin proficient (T4R) or deficient (T4RΔ*ply*) pneumococci. For dendritic cells and GM-CSF macrophages, we observed an inhibition of cytokine secretion (TNFα, IL-10 and IL-1β) by pneumolysin expressing pneumococci which could not be explained by pneumolysin induced cell death. Cytokine secretion by THP-1 derived macrophages, however, was activated in the presence of pneumolysin. M-CSF macrophages showed an intermediate phenotype with unaffected TNFα and IL-1β production and inhibited IL-10 production in the presence of pneumolysin. Cytokine secretion required bacterial uptake by all the cells and a mutant of the autolysin LytA induced similar effects as the pneumolysin mutant, indicating that the release of pneumolysin by autolysis might be important for its effects on the cells.

Since we found the most pronounced differences in the effect of pneumolysin between dendritic cells and THP-1 macrophages, we further investigated the role of pneumolysin in these cell types. We used siRNA to silence TLR4 in THP-1 macrophages and showed that the increased secretion of TNFα in response to pneumolysin expressing pneumococci did not require the presence of TLR4. Therefore, pneumolysin must activate THP-1 macrophages in a different manner and further studies are required to understand this activation. The cytosolic receptor NOD2 as well as STING can be activated by pneumococci in a pneumolysin dependent manner (64, 65, 67), and could be a possible explanation for the observed activation of THP-1 macrophages.

We explored the inhibitory effects of pneumolysin on dendritic cells by measuring the expression of 84 genes associated to innate immunity and TLR signaling. 29 of the genes were at least 2-fold up- or down regulated in dendritic cells infected with T4R or T4RΔ*ply* compared to uninfected cells. Interestingly, all cytokines were expressed higher in T4RΔ*ply*

infected than in T4R dendritic cells, apart from IFNβ, which was expressed to similar levels. We measured the secretion of IFNβ in dendritic cells infected with T4R and T4RΔ*ply* and confirmed that also the protein levels of IFNβ are unaffected by the expression of pneumolysin.

To investigate the fairly general inhibitory effects of pneumolysin on cytokine expression, we measured the expression of inhibitory proteins known to affect cytokine expression. We found an increased expression of *suppressor of cytokine signaling 1* (SOCS1) in dendritic cells infected with T4R compared to T4RΔ*ply* after 9 hours of infection and Western blot analysis showed that the SOCS1 protein level after 4 and 9 hours of infection also was higher in T4R than in T4RΔ*ply* infected DCs. SOCS1 inhibits JAK/STAT signaling by binding to interferon receptors as well as JAKs. We measured $STAT1_{Tyr701}$ phosphorylation in dendritic cells 3 to 7 hours after infection and found a delayed STAT1 phosphorylation in T4R compared to T4RΔ*ply* infected dendritic cells. Our data show that pneumolysin expression by pneumococci increases the SOCS1 levels in dendritic cells, leading to a delayed phosphorylation of STAT1, which might cause a general reduction in cytokine expression.

SOCS1 can also directly inhibit TLR dependent cytokine signaling by binding to TIRAP or the p65 unit of NFκB, which leads to their ubiquitination and degradation. An inhibition of NFκB regulated gene expression would inhibit the expression of most cytokines, while it would not affect IFNβ expression. Future studies will show if SOCS inhibits NFκB activation in dendritic cells infected with pneumolysin expressing pneumococci.

A pneumolysin dependent increase in SOCS1 levels has not been reported previously and none of the known functions of pneumolysin could explain the upregulation of SOCS. Therefore, we performed a pulldown to identify new interaction partners for pneumolysin. Next to 31 other proteins, we pulled down *macrophage mannose receptor 1* (MRC-1) from dendritic cell lysates but not from THP-1 macrophages. We confirmed the interaction between pneumolysin and MRC-1 by co-immunoprecipitation. Interestingly, the receptor is not expressed in THP-1 macrophages, whereas it is expressed in dendritic cells, GM-CSF and M-CSF macrophages.

MRC-1 is a lectin which with high affinity binds mannose and fructose. The receptor has no signal domain but it is important for non-opsonized phagocytosis. It has been shown that the receptor can mediate phagocytosis of pneumococci by binding to the capsular sugars (93, 94). Interactions between pneumolysin and MRC-1 have not been reported so far. However, pneumolysin can bind to sugars such as the blood antigen LewisX (153, 154), and structural analysis has shown that it can bind two mannose molecules (155). Interestingly, activation of MRC-1 has been connected to an anti-inflammatory phenotype (219) and binding of *Schistosoma* glycan to MRC-1 increases SOCS1 expression in dendritic cells and inhibits cytokine secretion (220). Only the cell types in which cytokine secretion was inhibited by pneumolysin expressed MRC-1. THP-1 macrophages did neither express MRC-1 or SOCS1. Future studies will show whether MRC-1 is connected to the inhibitory phenotype that we observe in dendritic cells.

To conclude, pneumolysin mediates differential effects on immune cells, ranging from an activation of cytokine secretion in THP-1 macrophages to an inhibition in dendritic cells. Future studies will unravel whether the ability of cells to express receptors such as MRC-1 or the expression of SOCS1 determines the effect of pneumolysin expression by pneumococci on cytokine responses.

4.3 PAPER III

Spatial representation and density of human factor H binding proteins on *Streptococcus pneumoniae* affects virulence function

Evasion of complement attack is an important survival strategy of *S. pneumoniae*. The pneumococcal surface protein PspC binds human Factor H and thereby prevents deposition of complement on the bacterial surface (174). While most pneumococci express PspC with a choline binding domain to anchor to the cell wall, a few PspCs are covalently link to the cell wall with a LPxTG motif. Our group previously identified clinical isolates of serotype 6B belonging to clonal complex CC138 which expressed two PspC proteins, PspC1 with a choline binding domain and PspC2 with a LPxTG motif (173).

Paper III compares the function of the two PspC proteins as well as their localization on the bacterial surface. We studied two 6B clinical isolates, BHN191 and BHN418. BHN191 was isolated from the blood of a meningitis patient, and BHN418 was a nasopharyngeal isolate of a healthy child. Flow cytometry showed that the ability of BHN191 and BHN418 to recruit Factor H to the bacterial surface was higher in comparison to D39 and T4 which only express one PspC protein.

Immunofluorescence staining of PspC1 and PspC2 as well as High resolution STED microscopy revealed that PspC1 and PspC2 bind to distinct parts of the bacteria. While PspC1 is localized at distinct para septal rings to the equatorial plane of dividing bacteria, PspC2 localizes at the poles of the bacteria.

Co-staining with Factor H showed that Factor H preferentially binds to sites not occupied by PspC2 with a pattern of septal rings similar to PspC1. Factor H staining of PspC1 and PspC2 deletion mutants in the BHN418 background, and analysis by Flow cytometry confirmed that PspC1 contributes more to Factor H binding than PspC2. This was in contrast to results from surface plasmon resonance where both copies bound Factor H equally well, indicating that the expression on the bacterial surface might affect the protein function.

Staining for C3 showed that both PspC proteins contribute to the protection from C3 deposition, but absence of PspC1 from the bacterial surface had a larger effect than absence of PspC2. The data indicate that PspC1 is the major Factor H binding protein of the two PspC proteins.

Next to Factor H binding and protection from C3 deposition, PspC is an important pneumococcal adhesin (180-182). We therefore assessed the role of the two PspC proteins in adhesion to A549 lung epithelia cells. Interestingly, we found that the absence of PspC2 significantly decreased adhesion to A549 cells, whereas the absence of PspC1 had no effect. Interestingly, fluorescence microscopy showed that the bacteria preferentially adhere to the cells via their poles, the area mainly populated by PspC2. Together this data indicate that PspC2 is the major adhesin of the two proteins.

Additionally, we assessed opsonophagocytic uptake of pneumococci by THP-1 derived macrophages. We found that uptake of serum opsonized pneumococci by macrophages was increased in the absence of PspC1, whereas the absence of PspC2 did not significantly affect uptake. This is consistent with the role of PspC1 as the major Factor H binding protein. However, a PspC1 PspC2 double mutant was phagocytosed less as compared to the PspC1 mutant. This might be explained by the role of PspC2 in promoting adhesion, a factor that could lead to decreased uptake in the double mutant.

Due to the distinct functions and distinct localization patterns of PspC1 and PspC2, we speculated that surface localization might contribute to the function of the proteins. Analysis of other LPxTG linked or choline binding PspC proteins showed that PspC is localized as septal rings in strains expressing only one copy of PspC, irrespective of the anchoring domain.

Previously it has been shown that the surface localization of proteins in Gram positive bacteria can be changed by altering the signal peptide (221).

A signal peptide switch mutant in which the signal peptide of PspC1 was exchanged with the signal peptide of PspC2 showed an altered localization pattern of PspC1 on the bacterial surface. Instead of distinct rings located at the septum, the mutant PspC1 distributed over a larger area. Due to the normal expression of PspC2, adhesion to A549 cells was not affected in the signal peptide-switch mutant. However, the ability of the mutant to bind Factor H was impaired, and C3 deposition was increased. Consequently, the opsonized mutant was taken up more efficiently by macrophages. Co-staining with Factor H revealed that Factor H binding did not fully overlap with the larger surface distribution of PspC1 on the signal peptide switch-mutant. Factor H bound to rings in the septal area where the density of PspC1 was the highest.

In summary, we found that the two PspC proteins PspC1 and PspC2 in serotype 6B strains belonging to CC138 fulfil distinct functions and are located differentially on the bacterial surface. While PspC2 localizes to the bacterial poles and is important for adhesion to epithelia cells, PspC1 is located as dense para septal rings and is the major Factor H binding protein. Changing the surface localization of PspC1 impaired its function and indicated that Factor H binding occurs at sites of high PspC1 density.

Serotype 6B strains belonging to CC138 are efficient colonizers of the nasopharynx in children but also successful in invasive disease (173, 200). The functions of PspC1 and PspC2 as Factor H binding protein and adhesin, respectively, might contribute to the success of this clone. It is interesting that adhesion and Factor H binding occur at distinct sites of the bacterial surface. Adhesive structures often are located at the apical poles of bacteria, such as the polar fimbriae of *Escherichia coli* (222), the polar presentation of ActA in *Listeria monocytogenes* (223) and the polar invasion of epithelia cells by group B streptococci (224). We found that C3 deposits fairly equally on the bacterial surface at sites not protected by PspC proteins, however it has been reported that the MAC complex preferentially assembles

at the division septum in *Streptococcus pyogenes* (225). Gram positive bacteria are protected from lysis by the MAC complex, which is believed to be due to the thick peptidoglycan layer of the cell wall (77), and the effect of MAC proteins binding to the septum is not known. However, the deposition of MAC proteins at the septum might be one reason for the septal localization of PspC1.

4.4 PAPER IV

Immunomodulatory effects of vitamin D on innate and adaptive immune responses to *Streptococcus pneumoniae*

Immunomodulatory effects of vitamin D are well documented (115-119). The effect of vitamin D on immune responses to pneumococcal infections however has not been investigated.

In paper IV we explored the modulatory effects of vitamin D on dendritic cell and T-cell responses to *S. pneumoniae*. Dendritic cells were stimulated with T4, the unencapsulated isogenic mutant T4R or pneumococcal *peptidoglycan* (PGN), and the expression of MHCII and the costimulatory molecule CD86 was measured. The anti-phagocytic capsule of T4 prevented upregulation of MHCII and CD86, whereas T4R as well as PGN triggered an upregulation of these maturation markers.

We studied the effect of the active form of vitamin D, 1,25 $(OH)_2D_3$, on dendritic cells and found that vitamin D enhanced CD86 expression in response to T4, T4R and PGN. Vitamin D did not affect CD86 expression on unstimulated cells. Pro-vitamin D, 25(OH)D3, was also able to induce a small increase of CD86 expression on PGN stimulated dendritic cells, and the effect of pro-vitamin D was blocked by itraconazole, an inhibitor of the Cyp27B1 1α-hydroxylase. Additionally, vitamin D upregulated the expression of the chemokine receptor CCR7 in response to PGN and inhibited uptake of T4R by dendritic cells.

The expression of the costimulatory molecule CD86 and the chemokine receptor CCR7, as well as reduced phagocytic activity, are typical changes associated with matured dendritic cells which migrate to the draining lymph nodes and present antigen. Our data indicate that dendritic cells are able to convert the pro-form of vitamin D into the active form, and that vitamin D enhances maturation of dendritic cells activated by pneumococcal components.

Further, the expression of the PGN receptors NOD2 and TLR2 was synergistically upregulated by vitamin D and PGN, whereas TLR4 expression was unaffected. Upregulation of the innate receptors TLR2 and NOD2 by vitamin D might be beneficial during pneumococcal infections since it can enhance the detection of bacteria by the immune system.

In line with the upregulation of the PRRs, expression of the antimicrobial peptide *human beta defensin 3* (hBD-3), and expression and secretion of IL-1β was increased in the presence of vitamin D. hBD-3 has antimicrobial activity against *S. pneumoniae*, and IL-1β is a pro-inflammatory cytokine important for the recruitment of phagocytes during infections. Increased killing by phagocytes and antimicrobial peptides can contribute to the clearance of pneumococcal infections.

Coculture of $CD4^+$ memory T-cells with PGN primed dendritic cells resulted in the secretion of large amounts of IFNγ and low amounts of IL-17 and IL-10. IFNγ, IL-17 and IL-10 are associated with T_H-1, T_H-17 and Treg phenotypes, respectively. The presence of vitamin D reduced IFNγ and IL-17 production and enhanced IL-10 secretion, indicating that the T-cell responses were skewed toward an anti-inflammatory phenotype by vitamin D.

Several studies showed an important role of T_H-1 and T_H-17 responses for the prevention of pneumococcal colonization and disease (100-106). The role of regulatory T-cell responses is less clear (108-110), but an anti-inflammatory T-cell phenotype might be beneficial for the prevention of excessive inflammation. While inflammatory responses are essential to clear pneumococcal infections, excessive inflammation and tissue destruction can promote pneumococcal disease. Further studies will provide insight into the clinical implications of vitamin D on pneumococcal infections.

In conclusion, vitamin D modulated the dendritic cell and T-cell responses to *S. pneumoniae*. The maturation of dendritic cells as well as the expression of key innate elements was enhanced by vitamin D whereas adaptive T-cell responses were dampened. Our data supports a possible positive effect of vitamin D on the human immune responses to pneumococcal infections, which will need to be confirmed by clinical studies.

5 CONCLUDING REMARKS

Even today, with the availability of vaccines and antibiotics, infections with *S. pneumoniae* remain a major health problem. For the development of future vaccines and treatment options a thorough understanding of the interactions between pneumococci and the immune system is essential. Macrophages are required for the immediate clearance of invading pneumococci and dendritic cells are essential for the initiation of appropriate adaptive responses. The cytokines secreted by dendritic cells determine the T-cell subtype which has significant effects on the immune responses as a whole.

In paper I we identified TLR3, a receptor previously not known to be activated by pneumococci, as a receptor for pneumococcal RNA in dendritic cells. The activation of TLR3 was essential for full secretion of the cytokine IL-12 and could be enhanced by prior infection with IAV.

Paper II explored the differential effects of pneumolysin on dendritic cells and macrophages. We found a cell death independent inhibitory effect of pneumolysin on dendritic cells and describe initial insight into the mechanisms behind this inhibition.

In paper III we discovered distinct roles in adhesion and complement evasion for the two closely linked proteins PspC1 and PspC2. The proteins were differentially localized on the bacterial surface, and correct localization was essential for the function of PspC1.

In paper IV we found that vitamin D enhances innate responses of dendritic cells to pneumococcal PGN, and modulates adaptive T-cell responses towards a regulatory phenotype. This effect of vitamin D on the immune responses might be beneficial during pneumococcal infection.

Effects of pneumococcal virulence factors cannot necessarily be transferred between cell types. Additionally, pneumococcal virulence factors can have multiple effects on the host and a slight disturbance of their surface expression can impair their function. This thesis underlines the complexity of the interplay between pneumococci and the host. The papers give insight into the activation (paper I and II), evasion (paper II and III) and modulation (paper IV) of the human immune responses to pneumococci. Hopefully, this knowledge will make some contribution to the development of protein vaccines or immunomodulatory therapies in the future.

6 ACKNOWLEDGEMENTS

First of all, I want to thank my Ph.D. supervisor **Birgitta Henriques-Normark** for these truly exciting and educational years. You allowed me to join your research group and introduced me to *Streptococcus pneumoniae*. During my time as a Ph.D. student I always felt that you fully supported and trusted me. I feel privileged to have worked with you and I know that I always will look back at this time with many great memories.

Thank you, **Laura Plant**, for being exactly the co-supervisor that I needed. Even though you left our group you were always available to me when I needed support.

Staffan Normark, discussions with you are always motivating and inspiring. Thank you for sharing your ideas with me.

It has been a pleasure working with all **co-authors** on my papers. Thank you for the good collaborations.

I want to thank all the past and present **BHN** group members as well as the **Rhen** and **Loh** group for a wonderful time. There are a few people that I want to specially address:

I want to thank **Marie**, for letting me join the vitamin D story which turned out to be my first publication ever.

Thank you **Anuj** for introducing me to the amazing world of PspC proteins and for letting me join your project. And of course, thank you, for letting me steal from your snack storage, for all the after work swimming in Brunsviken and for being the one who talks at lunch when everyone else is quite!

Vicky, thank you for our many discussions, for your help in various situations, your honest opinions and for joining me doing embarrassing exercises in Haga parken!

Karina, when I came for my interview, you had just started your Ph.D. studies with Birgitta. After talking with you, I knew I would have great company as a Ph.D. student in Birgitta's lab. It was fun to share this experience almost simultaneously with you. Thank you for discussing all my dissertation problems with me and for all your help!

"Lab" **Martin**, you made my Ph.D life easy! With you as my smart desk neighbor I could always get answers quicker than on Wikipedia. Thank you so much!

I never liked animal studies, but with you **Karin** and **Christel**, at least I had the best company!

Murat, **Ilias**, **Alice** and **Marilena**, you left our group quite a while ago, but I won't forget the fun times we had together, thank you!

All the people who joined our exciting trip to the ISPPD conference in India, thank you for this unforgettable experience! And thanks to Birgitta for letting us go ☺

The cream and cherry on top of my Ph.D. experience was the **EIMID-ITN** program. Thanks to all the EIMID fellows for a fantastic time. **Mario, Buket, Christina, Andy, Natalie, Laura** and **Alan**: thank you for all the fun on strange complementary skills courses all over Europe and all the great nights filled with Limoncello!

Next to my work life there were many people supporting me during these last years and I will just mention a few here.

Franzi, it was so fun to have you so close by in Uppsala, hope you move back to Sweden soon ☺

Thank you **Lumi**, for being such a good friend, you always cheer me up!

Tack, **David** och alla på svenka **språkkaféet** för en regelbunden distraktion från jobbet, och för förbättringen av min svenska. En färdighet som säkert kommer bli lika viktig för min karriär som doktorstiteln.

Hela Perssonfamiljen: tack för att ni välkomnade mig så varmt i eran familj. Ni är anledningen att jag känner mig så hemma i Svergie!

"My" **Martin**: If it wouldn't be for you I would never have ended up in Sweden doing a Ph.D.. Thank you for all the wonderful years together and for being at my side through all the ups and downs of my studies. You were my reason to come home from work and to stay away from the lab on the weekends.

Meine Familie ♡
Mama und **Papa**, danke dass ihr immer für mich da seid und mich in meinen Entscheidungen immer unterstützt habt! **Sarah** und **Franca**, ihr seid die besten Schwestern die ich mir wünschen könnte!

Thank you,
Danke,
Tack!

Laura

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